Monocyte responses to pro-atherogenic microenvironmental stimuli

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Declaration

The thesis has been composed by the candidate.

All work presented is the candidate's own.

The work has not been submitted for any other degree or professional qualification.

Jaydeep Sarma, February 2006

Preface

This is a resubmission of a thesis for the degree of Doctor of Philosophy. New work has been performed in accordance with the examiners' comments, and the text entirely restructured and revised.

The thesis assesses whether microenvironmental stimuli relevant to atherosclerosis alter monocyte survival, phenotype and gene transcription. These themes are emphasised through the text.

Thesis structure

The thesis commences with a general introduction, followed by methodology. Individual data chapters have further specific introductory, results, and discussion sections. The data presented is set into context, and follows the structure of the thesis. An overview of the thesis findings in the context of the current literature and future work is offered in the final chapter. The presentation of the thesis is in accordance with the University of Edinburgh postgraduate regulations with regard to character fonts, pagination, and formatting.

The following specific issues raised by the examiners have been directly addressed.

Introduction

The general introduction has been restructured, in the context of the principal research targets. The work presented is set in the context of the current literature documenting the general histology, cellular mechanisms and clinical sequelae of atherosclerosis, with a focus upon the distinction between early atherogenesis and advanced plaque progression. The thesis focuses upon the role of monocyte involvement in atherosclerosis, and upon the role that modified lipoproteins may play. However, further consideration is given to the wide range of other pathogenic factors and processes involved in atherogenesis. General mechanisms of inflammation are outlined, and the evidence suggesting a role for inflammation in atherosclerosis is reviewed. Specific mechanisms of arterial remodelling are described according to original references. The role that monocytes play in inflammation and vascular injury, and the kinetics of monocyte migration and sequestration at inflammatory sites are reviewed. The literature pertaining to monocyte apoptosis and survival is reviewed in the general introduction and in subsequent chapters, in the context of inflammation and atherosclerosis. Specific note is made of the influences of growth factors and inflammatory activation upon monocyte survival in the general introduction and in the relevant subsequent

chapters. The balance of cell survival and cell clearance in chronic inflammation is introduced, and is also reviewed in detail in subsequent chapters. Alternative strategies for investigating atherosclerosis including the use of animal models are discussed.

Methodology

Details of leukocyte isolation and culture are provided in full. Details of immuno-magnetic separation monoclonal antibodies are listed, along with specific details regarding suspension culture and the polytetrafluoroethylene containers used. Descriptions of apoptosis assays are provided, including details of blinding of observers during apoptosis assessment. Cell isolation and nucleic acid preparation for gene array studies are described in full. Details of light, fluorescence, con-focal and electron microscopy techniques, including cell preparation prior to image acquisition, are provided. Assessment of monocyte calcium flux is specifically described.

Data presentation

The order of chapters has been altered, with data on cell survival now presented first, followed by transcriptional profiling work and then monocyte surface phenotyping data. Micrographic evidence for lipid uptake by monocytes is presented. However, monocytes are referred to as lipid-laden, rather than using the specific term "foam cell". Details of monocyte purity are given, to demonstrate quality control during leukocyte isolation. The rationale for using reagents at the concentrations described is explained with reference to established literature in the field, and also by presenting data demonstrating concentration responses to the reagents used. The activity of specific agents used in studies has been formally assessed. Variation of monocyte responses over time and in differing culture conditions has been explored. Experiments have thus been repeated and extended for both apoptosis and phenotyping studies, to allow assessment of varying primary cell responses. Data regarding the transfection of monocytic cell lines are presented in a separate chapter. Transcriptional profiling data are presented in full, with data tables in the attached appendix.

Statistical analysis, sampling issues

The candidate has attended a course on medical statistics at the University of Edinburgh, and has received advice from professional statisticians regarding the use of tests for *in vitro* small sample number assays. The additional data for the experiments described have been analysed accordingly, with data analyses shown in the relevant appendices. The issue of population

variation in cell surface molecule expression and random sampling has been discussed, as the *in vitro* assays presented are not comparable to clinical studies or a clinical trial.

Discussion

The limitations of individual experiments, and potential studies that might extend the work presented, are discussed in individual relevant chapters. The data presented is critically assessed, and related to findings from other workers in the field. In particular, issues of flow cytometric analysis techniques and the use of appropriate controls have been discussed in detail. The concept of *in vitro* studies being artefactual and the use of alternative techniques including *in vivo* studies have been discussed. The use of transcriptional profiling in contrast with other assessments of cell function has been reviewed. A further overview of the work is given in a concluding chapter, with a consideration of implications for clinical disease and potential therapies.

Abstract

Atherosclerosis is a disease process that reduces arterial blood vessel calibre, and is a major cause of morbidity and mortality in the industrialised world. Decreased vascular flow reduces tissue viability, resulting in cardiac, cerebrovascular, renovascular and peripheral arterial diseases. Inflammatory leukocyte responses underlie atherosclerosis. Clinical epidemiological data show that pro-atherogenic factors, including hyperlipidaemia, uncontrolled hypertension and cigarette smoking increase vascular inflammation, elevating an individual's risk of developing atherosclerosis. Local inflammation may alter leukocyte phenotype, function and survival, activating resident atherosclerotic plaque macrophages, and exacerbating vascular injury.

This thesis aimed to assess whether monocyte survival, phenotype and gene transcription alters in response to pro-atherogenic and pro-inflammatory micro-environmental stimuli including oxidised low-density lipoprotein and cyclopentenone prostaglandins, metabolites of arachidonic acid.

Oxidised low-density lipoproteins and cyclopentenone prostaglandins accelerated monocyte apoptosis, in serum-free conditions, in a concentration dependent manner. Cyclopentenone prostaglandin-induced monocyte apoptosis was caspase dependent, but oxidised low-density lipoprotein-induced monocyte apoptosis only partially caspase-dependent. Monocyte apoptosis appeared to be independent of the nuclear receptor and transcriptional controller peroxisome proliferator receptor gamma PPARγ. Arachidonate-induced monocyte apoptosis appeared to be caused by a disruption of NF-κB mediated signalling. Monocyte apoptosis appeared inversely related to maturation, with naïve undifferentiated monocytes being more susceptible to programmed cell death than committed macrophages.

Monocyte transcriptional responses to oxidised LDL were assessed using gene mini-arrays. Genes for CD47 and CD11a showed significant levels of variation on the array, with CD11a changes being confirmed by polymerase chain reaction assessment.

Monocyte surface molecular changes induced by low-density lipoprotein were directly assessed by indirect immuno-labelling and flow cytometric analysis. Oxidised low-density lipoprotein elevated monocyte surface CD54 in early suspension culture, but then caused a down-regulation of CD54 in prolonged adherent culture. Oxidised low-density lipoprotein diminished monocyte surface expression of CD11b and CD11c in a manner dependent upon adhesion and maturation. CD49d expression appeared to be reduced by oxidised low-density lipoprotein in mature adherent monocytes. Early marginal reductions in CD11a expression were not seen in more mature monocytes.

Pro-atherogenic and pro-inflammatory micro-environmental influences thus appeared to regulate monocyte viability and gene transcription, although this is not necessarily reflected at a protein level. Monocyte surface phenotype appeared to alter following exposure to modified lipoproteins, in a manner that may cause changes in monocyte mobility. These findings may contribute to our understanding of cell death in atherosclerotic lesions, and potential limitations on cell mobility out of atherosclerotic plaque.

Commonly used abbreviations in this thesis

Apoprotein B100	apoB100
Apoprotein E	apoE
Adenosine triphosphate	ATP
Adenosine triphosphate binding cassette	ABC
Basic fibroblast growth factor	bFGF
Dendritic cell	DC
Deoxyribonucleic acid	DNA
Endothelial cell	EC
Fas activated death domain	FADD
Glycoprotein	GP
High density lipoprotein	HDL
Human leukocyte antigen	HLA
Immunoglobulin	Ig
I-kappa beta	I-kB
Interleukin	IL
Intercellular adhesion molecule	ICAM
Liver X receptor	LXR
Low density lipoprotein	LDL
Macrophage inflammatory protein	MIP
Macrophage colony stimulating factor	M-CSF
Major histocompatibility complex	MHC
Malondialdehyde	MDA
Monocyte chemoattractant protein	MCP
Native low density lipoprotein	n-LDL
Nuclear factor kappa beta	NF-κB
Oxidised low density lipoprotein	ox-LDL
Platelet endothelial cell adhesion molecule	PECAM
Platelet activating factor	PAF
Peroxisome proliferators activated receptor	PPAR
Phosphatidylserine	PS
Retinoid X receptor	RXR
Ribonucleic acid	RNA

Scavenger receptor	SR
T helper lymphocyte	Th
Toll-like receptor	TLR
Transforming growth factor	TGF
Tumour necrosis factor	TNF
TNF receptor activated death domain	TRADD
Trans-endothelial migration	TEM
Vascular endothelial growth factor	VEGF
Vascular smooth muscle cell	VSMC
Vascular cell adhesion molecule	VCAM
Very low density lipoprotein	VLDL

CHAPTER 1 PERSPECTIVES ON THE CELLULAR CONTRIBUTIONS TO ATHEROSCLEROSIS AND ITS COMPLICATIONS

Section A Introduction

Atherosclerosis is a progressive chronic inflammatory disease of large and medium calibre arteries. The accumulation of atherosclerotic plaque, a complex aggregate of cellular infiltration, altered extracellular matrix and lipid components within the arterial wall causes progressive or acute vascular occlusion (Davies 1996). Atherosclerosis places a large burden on health care systems, and cardiovascular diseases are currently the largest cause of death worldwide (Yach et al. 2004). Despite public health and pharmaceutical approaches to reduce risk factors including serum cholesterol concentrations (Scandinavian Simvastatin Survival Study investigators 1994), cardiovascular diseases remain the leading causes of death in the United States, Europe, and much of Asia (Braunwald 1997).

This thesis assesses microenvironmental influences upon peripheral blood monocytes that may promote atherosclerosis. The causes of cardiovascular disease and the natural history of atherosclerosis will be presented in this chapter. Basic concepts of inflammation will be introduced, and specific inflammatory mechanisms relevant to atherosclerosis will be considered. The role of the monocyte in inflammation will be reviewed in detail, with respect to survival and phenotype, and the control of gene transcription.

1.1 Cardiovascular risk

A combination of environmental risks including tobacco smoking (Villablanca, McDonald, & Rutledge 2000), hypertension (Dzau 1990) and genetic factors (Lusis, Mar, & Pajukanta 2004) increase the probability of developing clinical cardiovascular disease. Clinical manifestations of atherosclerosis increase with advanced age (Bilato & Crow 1996), and positively correlate to male gender (Schildkraut et al. 1989), increased body mass and central abdominal fat distribution (Bhatt et al. 2006). An increasing prevalence of obesity is causing a rise in diabetes and the metabolic syndrome, raising the risks of associated vascular damage in affected individuals (Dandona & Aljada 2002; Hak et al. 1999; Temelkova-Kurktschiev et al. 2002). Early clinical evidence demonstrated that elevated plasma cholesterol concentrations, especially low-density lipoprotein (LDL) cholesterol, constituted a major risk factor for accelerated atherosclerosis (Slack 1969). LDL reduction has been subsequently been associated with lower rates of clinical episodes in patients with documented coronary, cerebrovascular and peripheral arterial disease (Shepherd et al. 1995).

1.2 Histology of atherosclerosis: normal arterial structure

Atherosclerosis refers to specific histological changes within arterial walls, typified by lipid and cellular accumulation and arterial wall remodelling (Stary et al. 1995). The wall of normal arteries consists of an intimal layer lined by endothelium on the inner luminal aspect, and by elastic lamina on the outer aspect. The media, the muscular wall of the artery, lies abluminal to the intima, and is in turn surrounded by the densely collagenous adventitial layer (Figure 1.1).

1.2.1 Endothelium

The luminal contents of blood are separated from the arterial wall by an endothelial monolayer that acts as a high selectivity permeable barrier. A highly metabolic tissue lining the entire vascular tree, the endothelium is non-thrombogenic due to local prostacyclin production inhibiting platelet activation, whilst local plasminogen production promotes endogenous fibrinolysis (reviewed by Pearson 1994). Endothelial production of the vasodilator nitric oxide (Furchgott & Zawadzki 1980) balances with vasoconstrictors including endothelin, angiotensin and platelet-derived growth factor (PDGF), maintaining arterial tone (reviewed by Drexler & Hornig 1999). Endothelial cells (ECs) bear LDL receptors, facilitating LDL transport into the artery wall, an event associated with LDL modification (Kim, Dawes, & Jessup 1994). Endothelial injury is a primary trigger for atherogenic cellular responses (Rangaswamy et al. 1997) (see section B 1.5).

1.2.2 Intima

The normal intima is a thin basement membrane layer of connective tissue comprising of the matrix protein collagen IV and mixed proteoglycans, with occasional isolated vascular smooth muscle cells (VSMCs), (see section C 1.6). Collagen and elastin fibre deposition occurs during ageing, accompanied by concentric smooth muscle cell hyperplasia (Tracy 2001). The intima is the site of extracellular deposition of amorphous lipids and atherosclerotic plaque development (Stary 1992), a process accelerated in hyper-lipidaemic subjects (Simionescu et al. 1986).

1.2.3 Media

The arterial media consists of smooth muscle cells in a collagen and proteoglycan matrix (see section C 1.6). Medial smooth muscle cells form lamellar units, increasing in number proportional to arterial size, each sandwiched between fenestrated sheets of elastic fibres. Elastic internal and external laminae offer structural strength, whilst still permitting the transit of cells and molecules. Large calibre vessels with more than 29 concentric laminae

require their own vascular network, or vasa vasorum, to provide a blood supply to outer lamellae (Wolinsky & Glagov 1967).

1.2.4 Adventitia

The adventitia is a dense collagenous structure with collagen fibrils, elastic fibres and multiple fibroblasts. The adventitia is innervated, has lymphatic drainage and a separate vascular supply from the vasa vasorum. Novel microfibril glycoproteins have been recently identified that bind to elastin molecules, contributing to arterial strength (Toyoshima et al. 2005).

1.3 Gross histological change in atherosclerosis

Various pathological classifications have been used for atherosclerotic lesion grading. The American Heart Association (AHA) classification stages atherosclerosis according to histological composition, and further categorises phases of clinico-pathological progression (reviewed by Stary et al. 1994; Stary et al. 1995; Stary 2000). The phases, graded 1 to 5 describe the clinical activity of the lesion, from clinically silent, asymptomatic chronic occlusive and symptomatic angina to acute coronary syndromes. The histological stages grade lesions from I to VI according to cellular composition and histological structure, dependent upon the smooth muscle cell, macrophage and lipid composition (see Figure 1.2). Plaque complications including thin cap fibro-atheromata, and plaque erosions are not specifically included in this scheme, and have been addressed by other classifications (Virmani et al. 2000).

1.3.1 Early atherogenesis: Fatty streaks

Pre-atherosclerotic lesions are described as "fatty streaks." Evidence of fatty streaks in post-mortem studies of large arteries taken from infants and young children emphasises the early onset of atherogenesis (Napoli et al. 1997). Fatty streaks correspond to histological types I to III, part of the clinically silent Phase 1 of atherosclerosis (see Figure 1.2). Fatty streaks represent lipid-laden macrophages and smooth muscle cells, filled with cholesterol and cholesteryl ester, that have been transferred to the intima through the endothelial monolayer. A simplified view of fatty streaks is outlined in Figure 1.3.

1.3.1.1 Type I Lesions

Type I lesions have microscopically visible lipid droplets in the intima, and macrophage infiltrates. Hypercholesterolemia enhances monocyte adherence to the endothelium in porcine, (Gerrity 1981a) and non-human primate models of atherosclerosis (Faggiotto, Ross,

& Harker 1984). In the first 8 months of life, 45% of infants have coronary arterial macrophage foam cells, and a doubling of lipid-free macrophages (Stary 1987).

1.3.1.2 Type II Lesions

Type II lesions are visible as yellow streaks or spots on gross inspection. Lesional lipid content is readily labelled with fat-soluble dyes (Uemura 1964), and consists of cholesterol, phospholipids and cholesteryl oleate and cholesteryl linoleate esters (Katz, Shipley, & Small 1976). Macrophages increase in number further, appearing stratified rather than isolated. Intimal smooth muscle cells show intra-cytoplasmic lipid droplets, and T cell infiltration is notable (Munro et al. 1987).

Type IIa lesions preferentially progress to more advanced stages, typified by the increased presence of smooth muscle cells, intercellular matrix and deep intimal macrophage-derived foam cells and extracellular lipid droplets. Mechanical forces are associated with intimal thickening, increased lipid influx and early lipid accumulation in hyperlipidaemic subjects. Low shear stress prolongs LDL and arterial wall interactions increasing trans-endothelial LDL diffusion (Glagov et al. 1988). Type IIb lesions by contrast appear more resistant to lesion progression, and are associated with thinner intimal layers, and low levels of smooth muscle cell infiltrates.

The aorta, bifurcation points and ostia of large vessels, and the coronary arteries are associated with an increased likelihood of Type II lesion progression. 99% of children aged 2 to 15 years have type II lesions in the aortic arch, descending thoracic aorta, and abdominal aorta with further involvement throughout the length of the aorta from puberty (Strong & McGill, Jr. 1969) to the age of 20 years (Eggen & Solberg 1968). Type II lesions are seen in the coronary arteries by the time of puberty (Eggen & Solberg 1968), especially in the proximal 2cm of the left coronary artery (Montenegro & Eggen 1968). The intima of the proximal left anterior descending artery is particularly prone to Type IIa lesion formation in young adults (Stary 1989).

1.3.1.3 Type III Lesions

Also known as intermediate, transitional, or pre-atheromatous lesions, type III lesions have extracellular lipid droplets and pools distributed among smooth muscle cells in a thickened intima but no large lipid core is present (Stary et al. 1994). These lesions arise in the same progression-prone areas as Type IIa lesions and as more advanced lesions, suggesting they form part of a continuous spectrum of cellular change. Lipid pools lie below layers of macrophages and macrophage-derived foam cells, replacing intercellular matrix

proteoglycans, and drive smooth muscle cells apart (reviewed by Fuster, Badimon, & Badimon 1992).

1.3.2 Atherosclerotic lesion progression

Type IV lesions and lesions of greater complexity constitute the histological definition of atheroma. Type IV and Va lesions may be clinically silent (Phase 2 lesions), with lesions of type Vb, Vc and VI commonly producing clinical manifestations (see Figure 1.2).

1.3.2.1 Type IV Lesions

Type IV lesions have dense focal accumulations of extracellular lipid within the intima, termed lipid cores, a progression of the separate extracellular lipid pools in type III lesions, contributing to an atheromatous, or gruel-like plaque composition (see Figure 1.4). Type IV lesions are the first lesions considered advanced due to the marked intimal disruption that the lipid core causes (Stary 1989). Though non-stenotic, and classed as clinically silent Phase 2 lesions whilst quiescent (see Figure 1.2), these lesions are capable of rupture (Fuster et al. 2005). The intima appears thickened although atheroma may fail to narrow the vascular lumen, due to increases in the external boundary of the artery (Glagov et al. 1987). Intimal smooth muscle cells and deep intimal matrix are dispersed or replaced by accumulated extracellular lipid particles. Smooth muscle cell organelles may be calcified, and calcium particles are seen within the lipid core. The intima contains macrophages, and smooth muscle cells (with and without lipid inclusions) as well as lymphocytes and mast cells in the area between the lipid core and the endothelial surface (Jonasson et al. 1986). Populations of smooth muscle cells and collagen fibres are lower in this area in type IV lesions, and, along with high density macrophage and lymphocyte populations, contribute to structural weakness in the lesion periphery.

Capillary networks border the lipid core, particularly at the lateral margins and facing the lumen. Neo-vascularisation has been noted in advanced lesions. Vasa vasora infiltrate the adventitia and outer media but not intima of the aorta, coronary femoral and carotid arteries (Kwon et al. 1998), contributing to 70% of the capillary supply of a plaque, the rest being luminally derived. Micro-vascular infiltration in the base of lesions is associated with plaque degradation (Moreno et al. 2004).

Much of the tissue between the core and the surface endothelium corresponds to the proteoglycan-rich layer of the intima. A further increase in collagen content increases the fibrotic nature of type IV plaques. The upper intimal layer of a type IV lesion is indistinguishable from the fibrotic cap of a type V lesion, and both type IV and type V lesions are labelled fibrous plaque.

1.3.3 Complex atherosclerotic lesions; atherothrombotic events

1.3.3.1 Type V lesions

Type V lesions, or fibro-atheromata, have prominent fibrotic tissue. Medial smooth muscle cells are reduced in number and structurally disorganised. The media and adventitia contain macrophages, macrophage foam cells mast cells and lymphocytes, the latter in large groups near adventitial vasa vasorum (Jonasson et al. 1986) (see Figure 1.5). Type V lesions may be sub-classified.

Type Va lesions have multiple lipid cores, separated by thick fibrous connective tissue forming a multilayered fibro-atheroma. Fibrotic organisation of haematomas and thrombi is followed by further lipid-laden macrophage accumulation and extracellular lipid between fibrotic layers and the endothelial surface. Such lesions may be clinically silent (Phase 2), but may progress to Phase 3 or 4 with thrombotic complications (see Figure 1.2).

Type Vb lesions have calcified lipid cores and mineralised cell debris (Stary et al. 1992). Coronary calcification consists of hydroxyapatite crystals, and organic matrix calcification, including Collagen I and non-collagenous bone proteins, chiefly osteopontin (Fitzpatrick et al. 1994). Type Vc fibrotic lesions have no lipid core, minimal lipid content overall, and replacement of normal intima by fibrotic tissue. Often seen in large lower limb vessels (Ross et al. 1984), fibrosis is due to thrombus organisation, fibrotic extension from adjacent fibro-atheromata, or lipid core resorption. Type Vb and Vc lesions may cause clinical ischaemia (Phase 5). However, due to slow disease progression, collateral vessel development may protect target tissue resulting in clinically silent vessel occlusion, (see Figure 1.2).

Luminal encroachment of all type V lesions is greater than type IV lesions, but, as with type IV lesions, type V lesions may develop fissures, haematomata, and/or thrombus (progression to a type VI lesion, clinical Phase 3 or 4, see Figure 1.2).

1.3.3.2 Type VI complex lesions

Disruptions of the atherosclerotic lesion surface, haematoma or haemorrhage, and thrombotic deposits all classify a lesion as complex, or type VI, with the latter referred to as atherothrombotic events (see Figure 1.2). Although these lesions may be clinically silent they generally cause clinical symptoms and signs of ischaemia, and are sub-classified according to the type of lesional disruption, which varies in extent and severity. A schematic overview of these events is outlined in Figure 1.6.

1.3.3.3 Type VIa Surface Defects; Type VIb Haematoma

Microscopic surface ulcerations consist of focal endothelial cell loss; deep macroscopic ulcerations expose and release lipid from the lesion core. Type IV and Va lesions are prone to surface disruption, associated with high levels of inflammatory cell infiltration, proteolytic enzyme release by macrophages, coronary spasm and shear stress, and structural weakness related to lesion composition, (reviewed by Falk 1989). Fissuring is pronounced in areas of high density macrophage foam cell populations. In type VIb lesions, haematomas and thrombi are incorporated into lesions during fissure repair, accelerating plaque progression (Kolodgie et al. 2003) (see Figure 1.6).

1.3.3.4 Type VIc, thrombotic lesions

Advanced atherosclerotic lesions containing thrombi or thrombus remnants are common in humans from the fourth decade of life onwards. Thrombi vary in size from microscopic to grossly visible deposits, with some showing stratified layers suggesting repeated thrombotic deposition (see Figure 1.6). Hypercholesterolemia enhances platelet recruitment *via* adhesive interactions with endothelial cells, mediated by molecules including von Willebrand factor (vWF), glycoprotein Iba (GPIba), and P-selectin to lesion-prone sites (Theilmeier et al. 2002). Local release of tissue factor, a powerful pro-coagulant, further accelerates thrombus formation (Ardissino et al. 2001). Surface thrombi were noted in early studies of human atherosclerosis and more recently reported in advanced lesions seen in murine models of atherosclerosis (Rosenfeld et al. 2000). Thrombi may form over lesions with no gross surface disruption, through shear stress effects upon endothelial function at points of arterial angulation or flow division (Taeymans et al. 1992). Hyperlidaemia *per se* may promote endothelial vWF expression (Theilmeier et al. 2002) and platelet binding (Massberg et al. 2002) even in undisrupted lesions. Fissures and haematomas underlying thrombi may recur, inducing repeated formation of small thrombi. Lesion surface disruption greatly enhances thrombus formation, facilitated by local endothelial dysfunction and focal loss of small areas of endothelial cells (see Figure 1.6). Intra-lesional capillary haemorrhage may disrupt lesions to precipitate surface thrombosis, an event that may be associated with elevated secretion of proteolytic metalloproteinase enzymes (de Nooijer et al. 2006). Elevated plasma fibrinogen levels are seen in subjects suffering acute vascular occlusions, suggesting a systemic predisposition to thrombus formation (Woodward et al. 2003). Groups at high risk for atherothrombotic complications including cigarette smokers show exaggerated platelet aggregation in conjunction with elevated plasma fibrinogen levels (Fusegawa et al. 1999). Further enhancement of local thrombosis is caused by loss of natural endothelial fibrinolytic

mechanisms. Decreased fibrinolytic capacity is caused by increased levels of type 1 plasminogen activator inhibitor (de Bono 1994), and may be directly related to hyperlipidaemia. Lipoprotein(a), associated with high risk for clinical coronary heart disease, is structurally similar to plasminogen and may thus inhibit fibrinolysis by binding to fibrin or disrupting the assembly of fibrinolytic proteins (Loscalzo 1990).

Non-occlusive non-fatal thrombi that are not lysed contribute to increased lesion thickness (see Figure 1.6). Incorporation of such recurrent thrombi contributes to lesion bulk, with some thrombi enlarging and occluding lumina of medium-sized vessels within hours or days. Intimal smooth muscle cells invade thrombi and synthesise collagen, whilst the luminal surface of the thrombus is overgrown by endothelial cells. The presence of platelet-derived growth factor and platelet factor 4 contribute to smooth muscle cell proliferation, migration, and collagen synthesis (Pitsilos et al. 2003).

1.3.3.5 High-risk and vulnerable plaques

The progression of atheroma to complex stages is variable. More recent classifications have been devised to address the fact that advanced plaque progression to Type VI may occur rapidly and reflecting widespread vascular disease related to inflammation (Asakura et al. 2001). Early plaque, without a lipid or necrotic core but with marked local endothelial dysfunction may progress to thrombosis. Such plaque erosion occurs despite preserved smooth muscle cell and proteoglycan integrity, and the absence of matrix exposure (Schaar et al. 2004). Actual plaque rupture with thrombosis is usually associated with a lipid-rich core, and expansive compensatory remodelling. A thin fibrous cap covers rich macrophage and lymphocyte infiltrates but few smooth muscle cells, and is termed an inflamed thin cap fibroatheroma (Varnava, Mills, & Davies 2002). Plaques with calcified nodules also progress to thrombosis, and are characterised by heavy local calcification and luminal encroachment (Virmani et al. 2000). These additions to plaque classification correlate in more detail to acute clinical events, but complement the previous histological grading system.

1.3.4 Pathological and clinical sequelae of atherothrombosis

Atherothrombosis producing luminal encroachment may cause haemodynamic flow-limitation, inducing end-organ ischaemia in the myocardium, brain, major abdominal viscera or peripheral limbs (Virmani et al. 2000). If unresolved, or complicated by vessel occlusion, infarction and necrosis of susceptible tissue ensues, during ischaemic cerebrovascular accidents (Spagnoli et al. 2004) and during acute myocardial infarction (Falk, Shah, & Fuster 1995). Loss of end-differentiated neuronal or myocardial cells results in local fibrosis,

compromising organ function, with severe implications for patient morbidity and mortality (Collins et al. 2002).

Section B Inflammatory vascular injury

1.4 Overview of inflammation

Inflammation, the innate first response of the immune system to infection or cellular injury, is characterised by redness (*rubor*), heat (*calor*), swelling (*tumor*), pain (*dolor*) and dysfunction of the organs involved (*functio laesa*). Inflammatory cellular responses enable organisms to fight pathogens and limit severe infections (Anderson et al. 1985), while limiting cell damage and optimising cell recovery from mechanical and thermal injury.

Redness and heat are caused by the vasodilatation upstream of tissue injury sites, while local capillary permeability is increased, resulting in plasma extravasation and retention of water in hyaluronan-rich extracellular matrix (Mikecz et al. 1995). Downstream vasoconstriction increases oedema, distending and compressing nerve endings, causing pain. Stretch receptor mediated mast cell activation induces histamine and prostaglandin release. Activated mast cells also bind immunoglobulins *via* a repertoire of Fc receptors (Ra et al. 1989).

1.4.1 Inflammatory responses to primary cell injury

Classical inflammatory responses involve neutrophil recruitment to primary injury sites (Issekutz & Movat 1980). Excessive, unregulated inflammatory responses may be detrimental, causing inappropriate tissue destruction by proteolytic enzymes such as neutrophil elastase (Gadek et al. 1981). Cytokines, molecules that play a central role in local and systemic inflammatory responses, activate endothelial cells, upregulating adhesion molecule expression (see section 1.5, and Chapter 6 section 6.1.5), and aiding leukocyte extravasation from capillaries into tissue (Chuluyan et al. 1995). Cell motility is further enhanced in response to the CXC and CC chemokines, molecules that promote cell migration to inflammatory sites (del Pozo et al. 1995). Adhesion to activated endothelium activates neutrophils (Takahashi et al. 2001), priming them for a respiratory burst, producing high levels of hydrogen peroxide (Nathan 1987) enabling microbial destruction. Although tumour necrosis factor alpha (TNF-α) promotes NF-κB activation in endothelial cells, the co-existence of platelet-derived thromboxane $(A)_2$ may induce endothelial cell apoptosis, furthering vessel wall injury (Ashton et al. 2003).

1.4.2 Overview of cell death and phagocytosis in inflammation

Apoptosis or programmed cell death of extravasated leukocytes and their subsequent swift removal by phagocytes prevents excessive leukocyte accumulation at inflammatory sites (Haslett et al. 1994). Rapid phagocytic resolution of granulocyte influx is evident in clinical acute inflammatory diseases (Cox, Crossley, & Xing 1995). Phagocyte engulfment of apoptotic cells induces transforming growth factor-beta (TGF-β) inhibition of pro-inflammatory cytokine responses (Fadok et al. 1998a), although pro-inflammatory effects may be observed (Kurosaka, Watanabe, & Kobayashi 2001). Uncleared apoptotic cells may progress to secondary necrosis (Hebert et al. 1996), with resultant local pro-inflammatory consequences limiting reparative mechanisms. Necrotic cells may induce adaptive immune mechanisms by enabling dendritic cell maturation and cross-presentation of antigens to T lymphocytes thus prolonging inflammatory responses (Sauter et al. 2000). Chronic inflammatory responses to intracellular pathogens such as mycobacteria may cause granuloma formation by inhibiting normal phagosome function (Clemens & Horwitz 1995), or in the case of helminthic infections, by promoting anti-inflammatory cytokines (Ramaswamy, Kumar, & He 2000). The persistence of T-cells and monocytes are a feature of non-caseating granulomata in sarcoidosis, with reduced levels of apoptosis contributing to chronic inflammation (Xaus et al. 2003).

1.4.3 Inflammatory repair mechanisms

Restoration of function when inflammation subsides is dependent upon the regenerative capacity of injured cells. TGF-β1 plays multiple roles in inflammation, not only promoting monocyte chemotaxis, but also aiding resolution by increasing monocyte interleukin (IL)-1 production that in turn enhances fibroblast growth (Wahl et al. 1987). TGF-β directly promotes fibroblast production of fibronectin and collagen synthesis and incorporation into the extracellular matrix (Ignotz & Massague 1986), and regulates adherent leukocyte fibroblastic responses in an integrin dependent fashion (Munger et al. 1999). Fibrosis of specialised tissue composed of terminally differentiated cells is associated with functional loss. TGF-β2 directed responses provoke fibrosis following neuronal injury (Lagord, Berry, & Logan 2002). Cardiomyocyte replacement by collagen following myocardial infarction, (Colucci MD 1997) or auto-immune myocardial inflammation (Clancy & Buyon 2003) is associated with reduced cardiac ventricular function. IL-1α and IL-1β co-ordinate fibroproliferative responses by modulating growth factor production (Tamura et al. 1993). IL-1β directs fibroblast growth factor-2 mediated switching of collagen types in corneal injury, causing corneal opacification. In vascular structures, basic fibroblastic growth factor

(bFGF) and vascular endothelial growth factor (VEGF) contribute to arterial smooth muscle cell hyperplasia, diminishing vessel calibre (Lazarous et al. 1996).

Excessive fibrotic responses may thus limit recovery from injury by replacing damaged cells with matrix proteins, with implications in glomerulosclerosis (Stein-Oakley et al. 1997), pulmonary fibrosis (Huaux et al. 2003) and central nervous system inflammation (Logan et al. 1999) as well as in cardiovascular disease.

1.5 Specific inflammatory cellular mechanisms direct atherogenesis and atherosclerotic plaque progression

Atherogenesis was previously considered to be a passive process of lipid accumulation within the artery wall (Ross & Harker 1976). Subsequent data have suggested that lipid accumulation is only partially responsible for the development of atherosclerosis. The specific cellular and molecular responses underpinning the initiation and progression of atherosclerosis bear the hallmarks of an inflammatory disease (Libby, Ridker, & Maseri 2002). Studies in humans and animals suggest that atherosclerosis is a response to injury rather than simple lipid accrual, and it is reasonable to view atherosclerosis as a chronic inflammatory response to lipid retention (Hansson 2005).

Lesions may be initiated, regress or advance throughout an individual's lifetime, due to changing chronic inflammatory responses in the arterial wall. However, relatively small non-flow-limiting lesions may display elevated macrophage activation at vulnerable sites (Jonasson et al. 1986). Uncontrolled pro-inflammatory responses driven by local factors including CD40L ligation (Lutgens et al. 1999) may promote matrix metalloproteinase proteolytic enzymatic degradation of vascular wall components. The resulting exposure of matrix components may cause local thrombus deposition and thus vessel closure at sites of non-flow limiting, but inflamed plaque(Libby et al. 1996).

1.5.1 Inflammatory initiation of atherogenesis

Endothelial denudation, or endothelial cell layer loss, was previously thought to be the primary injury in the development of atherosclerosis (Bjorkerud & Bondjers 1973). More recent revisions of this model suggest that endothelial dysfunction rather than physical denudation of the endothelial monolayer is the critical initiating factor (see Figure 1.7). Key factors causing endothelial dysfunction include high concentrations of LDL, particularly modified LDL, and free radicals produced in response to cigarette smoking, hypertension, and glycosylation end-products in diabetes mellitus (Busse & Fleming 1996). Elevated plasma homocysteine concentrations, infectious micro-organisms such as herpes viruses or

Chlamydia pneumoniae, and combinations of these or other factors may also result in abnormal endothelial cell responses (Ross 1981).

Endothelial dysfunction promotes endothelial permeability (Hennig & Chow 1988) leukocyte-endothelial adhesion (Cybulsky & Gimbrone, Jr. 1991) and platelet-endothelial adhesion, permitting inflammatory responses to endothelial injury. The balance of pro-fibrinolytic endothelial properties is lost, with increases in vWF and reductions in endothelial cell production factors such as tissue plasminogen activator inducing a locally anti-fibrinolytic state (al Azhary, Wojta, & Binder 1994). High levels of angiotensin II further contribute to inflammatory endothelial cell injury (Weiss, Kools, & Taylor 2001), and augmented endothelial permeability to lipoproteins is enhanced by platelet-derived growth factor (Becker et al. 2000) and endothelin-1 (Schiffrin 2001).

The individual factors inducing endothelial injury relevant to atherosclerotic merit specific consideration.

1.5.2 Causes of endothelial injury: shear stress

Low haemodynamic shear stresses, and turbulent flow predict the anatomical sites of atheroma formation. Laminar shear forces are atheroprotective (Traub & Berk 1998), and disordered shear stress at vessel branch points, bifurcations and regions of high curvature are vulnerable to atherosclerotic plaque formation (Davies 1997). Cellular mechanotransducers include a complex containing the VEGF receptor-2 (VEGF-R2), the adherens junction proteins VE-cadherin and β-catenin (Shay-Salit 2002), and a complex composed of platelet endothelial cell adhesion molecule-1 (PECAM-1) in association with VEGF-R2 and vascular endothelial cell cadherin (Tzima et al. 2005). Transmitted shear stress from endothelial cell apices through cytoskeleton to cell-cell and cell-matrix adherens junctions induces the association of αvβ3 and β1 integrins with the adaptor protein Shc (Chen et al. 1999). Conformational activation of αvβ3 polarises endothelial cells in the direction of vascular flow, signalled by the small GTPases Rho (Tzima et al. 2001) and Rac1. The resultant NF-κB activation augments intercellular adhesion molecule-1 (ICAM-1/CD54) production (Tzima 2002) explaining earlier observations of this molecule in damaged endothelium (Nagel et al. 1994). Rheological influences alter the expression of endothelial cell genes with promoter regions responsive to shear stress (Topper & Gimbrone 1999). Expression of tissue factor on damaged endothelial cell surfaces is increased by shear stress (Lin et al. 1997), whereas endothelial cell-derived platelet-derived growth factor B chain is decreased by shear-stress, suggesting differential flow-related control of arterial wall remodelling in different areas of diseased vasculature (Resnick et al. 1993). Alterations in blood flow thus

critically determine which arterial sites are prone to atherogenesis (McMillan 1985) by regulating cellular recruitment signals to the endothelium.

1.5.3 Further causes of inflammatory vascular injury: Hypertension

Hypertension has pro-inflammatory effects, increasing the formation of hydrogen peroxide and free radicals such as superoxide anion and hydroxyl radicals in plasma (Lacy, O'Connor, & Schmid-Schonbein 1998; Morel, Hessler, & Chisolm 1983), substances that reduce endothelial cell production of nitric oxide, increase leukocyte adhesion, and increase peripheral arterial resistance. Concentrations of angiotensin II (AngII), a potent vasoconstrictor and principal product of the renin–angiotensin system, may be elevated in patients with hypertension, although this is not always apparent (Williams 2001). AngII may cause endothelial cell injury, and smooth muscle proliferation (Dzau 1993). AngII receptor-mediated activation of phospholipase C increases intracellular calcium concentrations, enhancing smooth-muscle contractility and hypertrophy (Gibbons, Pratt, & Dzau 1992). AngII also increases smooth-muscle lipoxygenase activity, which may increase LDL oxidation.

1.5.4 Further causes of endothelial and vascular injury: Homocysteine

Homocysteine, a sulphur containing amino acid, is a product of methionine catabolism and may alter oxidation-reduction balance within the arterial wall, with potentially toxic effects upon endothelial cells (Harker et al. 1976). Homocysteine promotes a pro-thrombotic state, (Hajjar 1993) increases collagen production (Majors, Ehrhart, & Pezacka 1997) and decreases local nitric oxide availability (Upchurch, Jr. et al. 1997). Homocysteine may upregulate apoB oxidation and increase monocyte activation and platelet aggregation. Elevated plasma homocysteine concentrations are linked with advanced atherosclerosis and severe early-onset atherosclerosis in subjects bearing homozygous defects in homocysteine metabolism enzymes, such as cystathionine beta-synthase or methylenetetrahydrofolate reductase (McCully 1993; Nygard et al. 1997). Plasma homocysteine concentrations are also slightly elevated in many subjects with no enzymatic defects in homocysteine metabolism, but with an increased risk of coronary, peripheral and cerebral atherosclerosis. Folic acid supplementation may reduce plasma homocysteine concentrations to normal (Omenn, Beresford, & Motulsky 1998). However, recent data suggest that despite homocysteine reduction, folic acid supplementation may not substantially alter vascular disease progression (Liem et al. 2003), arguing that homocysteine elevation may be a marker rather than a mediator of continuing vascular injury.

1.5.5 Further causes of endothelial and vascular injury: infection

Studies have suggested an association between atherosclerosis and at least two infectious microorganisms: herpesviruses and *Chlamydia pneumoniae* (Hendrix et al. 1990; Jackson et al. 1997; Libby, Egan, & Skarlatos 1997). Both organisms have been identified in atheromatous lesions in coronary arteries at post-mortem (Gupta et al. 1997a), and increased antibody titres of these organisms have been used to predict the occurrence of further adverse events in patients who have had a myocardial infarction. However, there is to date no direct evidence that these organisms are directly causative for atherosclerotic plaques (Hajjar et al. 1986; Nicholson & Hajjar 1998). Although these organisms are seen in many tissues and organs, the fact that lesions cannot be induced experimentally in animals by infection with these organisms leaves their role as aetiologic agents in question. Recent data suggests that respiratory tract infections are associated with a higher incidence of cerebrovascular accidents and myocardial infarction (Smeeth et al. 2004). It is possible that a protracted inflammatory stimulus derived primarily from a single or repeated infection, in the context of other secondary factors, may be responsible for atherogenesis or atherosclerotic progression in some subjects (Danesh, Collins, & Peto 1997).

1.5.6 Hyperlipidaemia, modified lipoproteins & inflammatory vascular injury

A key cause of endothelial dysfunction in atherosclerosis is systemic dyslipidaemia. LDL may be modified by oxidation, glycation in diabetes, direct lipid aggregation, association with proteoglycans, or incorporation into immune complexes (Khoo et al. 1988; Khoo et al. 1992; Navab et al. 1996; Steinberg 1997). Altered lipoproteins not only cause endothelial injury but also affect underlying smooth muscle cells (Griendling & Alexander 1997). LDL particles undergo progressive but variable oxidation in the vessel wall with the initial production of minimally modified LDL (mmLDL, see Figure 1.7) subsequently followed by ox-LDL species (Liao et al. 1991). LDL uptake *via* LDL receptors (LDL-R) results in homeostatic downregulation of LDL-R surface density (Kita et al. 1982). Modified LDL is internalised by macrophages *via* surface scavenger receptors, an alternative route for lipid intake, leading to further intracellular oxidation (see Figure 1.8), with lipid peroxide formation facilitating the accumulation of cholesterol esters (Han et al. 1997; Morel, Hessler, & Chisolm 1983). Removal and sequestration of modified LDL may represent an important part of the initial protective role of the macrophage in minimising the toxic effects of modified LDL on endothelial and smooth-muscle cells (Falcone, McCaffrey, & Vergilio 1991; Han et al. 1997). Antioxidants such as vitamin E may reduce the capacity for free-radical formation by modified LDL (Nunes et al. 1997). Modified LDL has been reported to

be chemotactic for monocytes and can augment transcription of genes for macrophage colony-stimulating factor (Quinn et al. 1987) and monocyte chemotactic protein (Leonard & Yoshimura 1990) derived from endothelial cells. It might be postulated that LDL acts to expand the inflammatory response by promoting the entry of circulating monocytes into lesions increasing the number of monocyte-derived macrophages present within atherosclerotic plaque.

Sub-particles within LDL, in particular apoprotein B100, trigger inflammatory responses. Binding between atherogenic lipoproteins and proteoglycans is mediated *via* ionic interactions between basic amino acids in apoB100 and negatively charged sulphate groups on the proteoglycan components of the arterial extracellular matrix. Mice expressing proteoglycan-binding-defective LDL develop significantly less atherosclerosis than mice expressing wild-type control LDL, suggesting that sub-endothelial retention of apoB100 is critical in atherogenic initiation (Skalen et al. 2002).

Inflammatory mediators including tumor necrosis factor-α, IL-1 and M-CSF, promote LDL binding to endothelium and smooth muscle and increase LDL-receptor gene transcription (Hajjar & Haberland 1997; Stopeck et al. 1993). Scavenger receptor binding to modified LDL *in vitro*, initiates a cascade of intracellular events that include the production of urokinase (Falcone, McCaffrey, & Vergilio 1991) and inflammatory cytokines including IL-1 (Geng & Libby 1995; Palkama 1991; Palkama, Matikainen, & Hurme 1993). Modified LDL thus promotes local vascular inflammation at sites of vascular injury.

Ox-LDL has been directly identified in human atherosclerotic lesions (Ylä-Herttuala et al. 1989). A role for ox-LDL in the pathogenesis of atherosclerosis has been further strengthened by evidence from studies of antioxidant therapies. In animals with hypercholesterolaemia, antioxidants can lower adhesion molecule expression. (Fruebis et al. 1997) and reduce the size of atherosclerotic lesions, (Carew, Schwenke, & Steinberg 1987; Chang et al. 1995; Griendling & Alexander 1997; Kita et al. 1987) and fatty streaks in non-human primates (Sasahara et al. 1994). Antioxidants increase the resistance of human LDL to oxidation ex vivo (Reaven et al. 1993) in proportion to the vitamin E content of the plasma. Vitamin E intake was found to be inversely correlated with the incidence of myocardial infarction, and vitamin E supplementation was found to reduce coronary events in a preliminary clinical trial (Rimm et al. 1993; Stampfer et al. 1993; Stephens et al. 1996). However, other antioxidants, such as beta carotene, have no benefit, (Hennekens et al. 1996; Omenn et al. 1996) and recent clinical data have failed to confirm any significant clinical benefit from Vitamin E supplementation(Collins et al. 2002) questioning the role of systemic anti-oxidant therapies in vascular disease.

1.5.7 Cellular lipid trafficking in inflammatory cells

Lipoprotein trafficking within mammalian vasculature is controlled at the level of absorption, hepatic processing and uptake by target cells, including adipocytes under the control of orphan nuclear receptors (Schoonjans, Staels, & Auwerx 1996) and leukocytes within atherosclerotic plaque (Hajjar & Haberland 1997). Ingested fatty acids from dietary sources cross the gut wall *via* active transport mechanisms in the form of chylomicrons: large particles with a high fat to protein ratio (Hussain et al. 1996). After being directed towards the liver (Kita et al. 1982), chylomicrons undergo specific degradation steps. The residual remnant lipoproteins are also pro-atherogenic, altering endothelial cell adhesion molecule expression (Doi et al. 2000) and inducing foam cell phenotypic changes in monocytes (Saito et al. 1996; Yu & Mamo 2000). Smaller, denser lipid-laden particles are also produced with higher protein to lipid ratios, and are classified by density. ApoB48-containing very low density (VLDL) (Luchoomun et al. 1997) bears a high triglyceride content, whilst apoB100-rich LDL has a higher protein to lipid ratio, the specific composition of which has been correlated to vascular disease (Capell et al. 1996). Epidemiological observations further demonstrate a relationship between atherosclerosis and elevated LDL levels (Gordon et al. 1981). High density lipoproteins (HDL) with lower lipid to protein ratio, are implicated in reverse cholesterol transport and cholesterol efflux (see Figure 1.8) involving the ATP binding cassette (ABC) transport mechanism a group of evolutionary highly conserved cellular transmembrane transport proteins that govern trans-membrane molecular trafficking (Aiello, Brees, & Francone 2003). HDL levels are inversely related to adverse cardiovascular clinical events (Mertens & Holvoet 2001). HDL effectively clears lipid from tissues during normal cholesterol trafficking *via* ABC-1 transporters, explaining the clinical observation that elevated HDL:LDL ratios limit atherosclerotic disease sequelae even if high total lipid levels remain elevated (Navab et al. 1995). Mutation of an ABC family member, the ATP-binding cassette A1 (ABCA1), causes Tangier disease (Rust et al. 1999), producing hypersplenism, macrophage accumulation and deposition of cholesteryl esters in the reticuloendothelial system, low plasma HDL and premature atherosclerosis. ABCA1 mRNA and protein levels are upregulated by uptake of modified LDL and downregulated by HDL-mediated lipid efflux in macrophages, suggesting that local lipoprotein species may directly affect inflammatory leukocyte function in atherosclerosis (Hovingh et al. 2003; Oram & Lawn 2001).

1.5.8 Inflammatory processes in fatty-streak formation

Macroscopic infiltration of fatty streaks by lipid-laden monocytes and macrophages together with T lymphocytes (Stary et al. 1994) contributes to the early initiation of inflammatory responses to lipid retention (Libby, Ridker, & Maseri 2002) (see Figure 1.8). T-cell association with macrophages in the setting of elevated levels of TNF-α and IL-2 is visible in early atherosclerotic plaque (Kishikawa, Shimokama, & Watanabe 1993). T-lymphocyte activation mediated by CD40/CD40 ligand interactions (Buchner et al. 2003) contributes to altering local cytokine responses within an evolving lesion, affecting local monocyte or monocyte-derived macrophages (see Figure 1.9). Monocytes within fatty streaks are exposed to pro-inflammatory input from oxidised LDL (ox-LDL), monocyte chemoattractant protein-1 (MCP-1) (Takeya et al. 1993) and IL-1β (Kirii et al. 2003). TNF-α, although pro-inflammatory, may act to protect against early atheroma by reducing macrophage scavenger receptor expression, limiting lipid ingestion (Schreyer, Peschon, & LeBoeuf 1996). Early vascular smooth-muscle cell (VSMC) migration is stimulated by PDGF in a MAP-kinase dependent manner (Graf et al. 1997).

1.5.9 Cell kinetics of inflammation in atheroma

Inflammatory mechanisms involve the ordered recruitment of leukocytes to facilitate efficient resolution of cell damage. Following primary cell injury in most tissues neutrophil infiltration enables the phagocytic engulfment of infectious organisms, and release of cytotoxic granulocyte contents (Haslett, Savill, & Meagher 1989). Polymorphonuclear cells undergoing programmed cell death are cleared by infiltrating macrophages that act to dispose of granulocyte debris whilst minimising local inflammatory responses (Meagher et al. 1992). Macrophages then emigrate to draining lymph nodes, where antigen presentation of pathogens may occur (Bellingan et al. 1996). Atherosclerotic plaque is dynamic, continually recruiting new inflammatory cells, and altering structurally in a response to chronic inflammation. Compensatory outward vascular remodelling counteracts initial luminal loss by an increase in arterial luminal diameter, involving matrix degradation, cellular division and hyperplasia and fibroproliferative repair responses, so that the effective luminal diameter remains unaltered (Glagov et al. 1987; Korshunov & Berk 2003). Although this process may allow for maintenance of intra-luminal blood flow, further increase in atheroma plaque bulk may ultimately contribute to reduced blood flow rates. Cell dynamics in atheroma have been described using *in vitro* models and also *ex vivo* histological samples in an attempt to address the temporal sequence of cell infiltration into atheroma (Kling, Holzschuh, & Betz 1993).

1.5.10 Role of granulocytes in inflammatory vascular injury

Although monocytes and lymphocyte infiltrates are well documented in histological descriptions of atherosclerosis, the involvement of granulocytes was rarely reported in early analyses of atherosclerotic plaque (Ross & Glomset 1976). The absence of neutrophils would be unusual for a classical inflammatory response and neutrophil infiltrates have indeed been reported in experimental models of atherosclerosis (Trillo 1982). Subsequent evidence has highlighted neutrophil interactions with endothelial cells (Topham et al. 1998) and responses to vascular trauma (Welt et al. 2000) relevant to arterial disease. Neutrophil recruitment to areas of vascular injury may alter local repair mechanisms, particularly acting to promote neointimal growth at sites of vascular trauma (Bienvenu et al. 2001). Neutrophils may be recruited to arterial injury sites by at points of endothelial dysfunction (see Figure 1.7), or by binding locally sequestered platelets (see Figure 1.12). Downstream signalling from platelet GPIbα to neutrophil Mac-1 integrin may then increase neutrophil activation promoting local tissue injury (Simon et al. 2000a).

Previous studies reporting an absence of intra-plaque granulocytes may not have visualized lesions at the precise time of granulocyte infiltration. Clinical studies and animal models suggest the absence of neutrophils, or components of neutrophil mediated inflammation, may reduce atherosclerotic initiation and progression (Kawaguchi et al. 1996; Provost & Merhi 1996). Neutrophils further regulate atherothrombosis by enhancing monocyte tissue factor production, indirectly promoting thrombosis at sites of monocyte infiltration within atherosclerotic plaque (Cadroy et al. 2000).

Granulocytes have not been frequently reported in atherosclerotic lesions beyond an initial injury phase (Kling et al. 1987), contrasting with the chronic inflammatory infiltrates in glomerulonephritis (Naish et al. 1975), rheumatoid arthritis (Mohr & Menninger 1980) and pulmonary fibrosis (Jones et al. 1998). In arthritis, granulocyte infiltration of the joint cavity is an initial feature. Macrophage and lymphocyte invasion of synovial cavities then contributes to cartilage and bone erosion (Jensen & Christensen 1990), leaving fibrous pannus (Chu et al. 1991). In pulmonary fibrosis, granulocytes initially appear in the alveolar spaces; however, the lung parenchyma, where fibrosis ultimately occurs, is infiltrated by macrophages and lymphocytes (Krombach et al. 1996). Thus, there are parallels between atherosclerosis and these other inflammatory diseases, albeit in the more chronic phases of disease progression.

Section C Complications of atherothrombosis

1.6 Matrix and smooth muscle cells in vascular inflammation responses

Atherosclerotic lesion complexity is added to by the continued recruitment of VSMCs, mediated by specific growth factors, (see section 1.18, and Figure 1.9). TGF-ß enhances VSMC fibroproliferative reponses increasing lesion bulk (Ma et al. 2000) and contributing to fibrous cap formation (Figure 1.9 and 1.10). PDGF regulates VSMC production of fibroblast growth factor-2 (Midgley & Khachigian 2004), suggesting that platelet-derived stimuli may contribute to lesion expansion and organisation (Figure 1.11). Smooth-muscle cells in the media of arteries, and in atherosclerotic lesions, are surrounded by different types of connective tissue (see Figure 1.10). In arterial media the matrix consists of type I and III fibrillar collagen, whereas in the lesions of atherosclerosis biglycan and decorin proteoglycans predominate intermixed with loosely scattered fibrils of collagen types I and III (Riessen et al. 1994). When cultured human arterial smooth-muscle cells are plated on collagen in fibrillar form, the collagen inhibits cell proliferation by up-regulating specific inhibitors of the cell cycle (Koyama et al. 1996). *In vivo* degradation of the collagen by collagenase, or migration away from this inhibitory environment, may allow smooth-muscle cells to respond to mitogenic stimuli and replicate, as they do when they are cultured on non-fibrillar, monomeric collagen. Other matrix molecules, such as fibronectin and heparan sulphate, may also be involved, because they have also been shown to inhibit the cell cycle, and cell–matrix interactions can lead to the induction of chemokines (Assoian & Marcantonio 1996; Mercurius & Morla 1998; Wesley, II et al. 1998).

The influence of matrix microenvironment on macrophage behaviour within plaque is less well defined. Matrix interactions have been shown to influence cellular production of reactive oxygen species, enzymes and cellular survival, with profound implications for inflammatory and fibroproliferative responses (Smith et al. 1997). Effects of monocyte activation upon matrix proteins regulate atherosclerotic plaque structure. Monocyte-derived matrix-metalloproteinase (MMP) activity compromises vessel wall integrity by degrading extra-cellular matrix (Galis et al. 1994) (see Figure 1.11). Exposure of extra-cellular matrix components to intra luminal blood promotes additional local MMP-9 production, enhances local tissue factor production and contributes to *in situ* thrombus formation (Morishige et al. 2003). Smooth muscle cells produce tissue factor themselves, following CD40 ligation (see Figure 1.9), accelerating thrombus formation in vascular injury (Schonbeck et al. 2000).

1.7 Platelets and vascular injury

Platelet adhesion and activation are important in animal and human atherogenesis. Platelets can adhere to dysfunctional endothelium, exposed collagen, and macrophages. When activated, platelets release granule contents containing cytokines and growth factors that, together with thrombin, may contribute to the migration and proliferation of smooth-muscle cells and monocytes (Bombeli, Schwartz, & Harlan 1998). Activation of platelets induces arachidonic acid formation, which can further be transformed into prostaglandins including the vasoconstrictor and platelet-aggregator thromboxane A2, or into leukotrienes which can amplify the inflammatory response (Matthias 1997).

Plaque rupture and thrombosis are notable complications of advanced atherosclerotic lesions that lead to acute vascular occlusion (Ross 1993). Platelets are physiologically important in maintaining vascular integrity and protecting against spontaneous haemorrhage (Harker & Ross 1978). Activated platelets can accumulate on the arterial walls and recruit additional platelets into an expanding thrombus (see Figure 1.11). The integrin GPIIb/IIIa receptor appears on the surface of platelets during platelet activation and thrombus formation (Coller 1985, see Chapter 6 section 6.1.6.2). However, increased platelet aggregation may be detrimental in vascular injury, and GPIIb/IIIa antagonists aid the prevention of intra-vascular thrombus formation in patients suffering from acute coronary syndromes (ACS) (Goodman et al. 2003). Platelet-leukocyte signalling may be important in promoting leukocyte activation (see Figure 1.12), and is elevated in ACS (Sarma et al. 2002). Platelet derived components including platelet activating factor (PAF) and CD40 ligand may activate macrophage protease secretion, resulting in further acute plaque matrix degradation (May et al. 2002), and platelet leukocyte interactions are associated with neo-intimal proliferation (Wang et al. 2005).

1.8 Immune responses in plaque progression and instability

The majority of myocardial infarctions occur as a result of erosion or uneven thinning and rupture of the fibrous cap, at macrophage-rich shoulders of the lesion. An immunoregulatory molecule, CD40 ligand (Hollenbaugh et al. 1995), can be expressed by macrophages, T cells, endothelium, and smooth muscle in atherosclerotic lesions *in vivo*, and its receptor, CD40, is expressed on the same cells. Activated T cells stimulate macrophages to release IL-1β and increase MMP production *via* CD40/CD40L dyad mediated signalling (see Figure 1.9) enhancing the inflammatory responses within lesions and promoting plaque instability (Schonbeck et al. 1997). CD40-mediated signalling promotes tissue-factor production (Bavendiek et al. 2002; Mach et al. 1997) leading to thrombosis. Inhibition of CD40 with

blocking antibodies reduces lesion formation in apolipoprotein E–deficient mice (Mach et al. 1998) supporting a role for this molecule in atherosclerosis. Although experiments with mice bearing gene deletions for both apo-E and recombinase activating gene-1 Rag-1 demonstrated that lymphocytes are not required for atherosclerotic plaque formation, it appears that they contribute to inflammatory injury in established lesions (Dansky et al. 1997). Th1 lymphocytes produce interferon gamma, inhibiting smooth muscle cell collagen production and smooth muscle cell proliferation (Gupta et al. 1997b), limiting fibroproliferative repair. Together, these findings implicate adaptive immune responses in the development of unstable atherosclerotic plaque.

Section D The role of monocytes in orchestrating atherosclerosis

1.9 Monocytes in Inflammatory Vascular Injury

Monocytes are crucial in the inflammatory cascade that follows cellular injury. Monocyte phagocytic function is central to disposal of cellular debris and the ingestion and clearance of pathogenic material (Haslett 1992; Savill et al. 1993). In addition, monocytes help direct reparative changes at the lesion sites. Monocytes contribute to the innate immune response the resolution of inflammation and the initiation and regulation of acquired immune responses. Monocytes adopt diverse roles in these fundamental processes with external influences, including cell-cell interactions during transmigration, regulating the high-level of monocyte phenotypic and functional plasticity (Gordon, Keshav, & Chung 1988). The transition of monocytes to tissue-specific differentiated cells is a response to specific trafficking and migratory stimuli (Randolph et al. 1998) integrated with local inputs from chemical (Mohty et al. 2003) and autocrine signals (Brewington et al. 2001). Disparate triggers may thus produce uniquely tailored responses peculiar to individual pathological insults, correlating with the high diversity of *in vitro* monocyte differentiation (Golub & Pagan 1986; Lyakh et al. 2000; Ruppert et al. 1991; Young, Lowe, & Clark 1990). In atherosclerosis, local tissue injury at the level of the endothelium (although not necessarily endothelial denudation (Joris et al. 1983)) results in recruitment of circulating intra-vascular monocytes by promoting monocyte margination to the vascular wall (Cybulsky & Gimbrone, Jr. 1991). Subsequent differentiation following transendothelial migration is further modulated by contact with resident cells, matrix components (Rekhter et al. 1993), and chemokine and cytokine signals (Sugiyama et al. 2001). The role of lipids has been of key interest in the development of this model. Human post mortem specimens demonstrate elevated lipid infiltration at heavy plaque burden sites within the arterial tree (Faggiotto &

Ross 1984), and suggest that monocyte handling of lipid is crucial to atherosclerotic plaque progression (Adams, Bayliss, & Turner 1975; Smith et al. 1995).

1.10 Monocyte Origin

Monocytes are myeloid-derived leukocytes that act specifically early in innate immune responses. Early myeloid precursors may differentiate along multiple pathways (see Figure 1.13) including a granulocyte developmental programme, or a monocytic programme (Nichols & Weinberg 1989) determined by critical transcriptional changes (Orkin 1995; Shivdasani & Orkin 1996). Basophils and eosinophils also originate from this precursor, although the latter cell type may undergo a separate differentiation pathway (Lawson & Berliner 1998). Bone marrow stromal maturation signals drive differentiation programmes influenced by systemic levels of cytokines including GM-CSF and IL-4 (Keisari et al. 2000), and colony stimulating factor-1 (CSF-1) (Serreze, Gaedeke, & Leiter 1993), altering the balance of myeloid cell production. Myeloid progenitor to monocyte progression is irreversible (Akashi et al. 2000), but monocytes are not entirely terminally differentiated. Further phenotypic variation denoting differential maturation (Dransfield et al. 1988), and response to environment (Freedman et al. 1991), with implications for functional specificity have been shown. The acquisition of specific phagocytic receptor repertoires (Fadok et al. 1992; Fadok et al. 1998a; Savill et al. 1990; Wahl et al. 1992), or differentiation into specific antigen presenting cells (Pickl et al. 1996) is of particular interest. Sub-specialised or altered monocyte phenotypes may be found in a number of pathological conditions, and may range from multi-nucleate giant cells through Kupffer cells in the liver, to foam cells in the atheromatous plaques of diseased arterial vessels. Recent attention has been drawn to phenotypically distinct pro-inflammatory monocyte subsets with characteristic low CD14 expression and high CD16 levels (Fingerle et al. 1993). Although only comprising a small percentage of total monocyte numbers, such monocyte subsets may disproportionately contribute to inflammatory responses.

1.11 Monocyte characteristics

Monocytes are typically in the order of 7-10 micron in diameter (Arenson, Jr., Epstein, & Seeger 1980). Morphologically they have large ovoid nuclei classically bearing a central indentation, with very densely packed and clumped chromatin (van Furth, Raeburn, & van Zwet 1979). In contrast to lymphocytes, monocytes have a relatively high cytoplasmic to nuclear ratio. The cytoplasm often vacuolates during monocyte differentiation, and this appearance may become marked during the progression towards a functional macrophage phenotype (Edelson & Cohn 1974; Lotan, Sharon, & Goldman 1977). Monocytes bear

markers including CD13 and CDw123 (Leveque et al. 1998; van Hal et al. 1992) derived from and denoting their myeloid lineage in marked distinction to lymphocyte precursors which classically bear markers including CD10, CD34 CD38 CD127 and HLA-DR (Bender et al. 1994; Plum et al. 2000; Tazzari et al. 1987; Terstappen, Huang, & Picker 1992). By contrast, HLA-DR becomes more evident in monocytes during maturation (Te Velde et al. 1988). The progression from pre-monocyte to monocyte introduces a significant increase in cell surface markers (Allavena et al. 1998), with further changes apparent during maturation to a macrophage phenotype. CD16 (low-affinity Fc receptor) and CD51 (vitronectin receptor alpha chain) are only expressed after serum-induced macrophage differentiation (Andreesen et al. 1990).

1.12 Monocyte Kinetics

Monocytes spend a limited time within the bone marrow following production and are rapidly seen entering the intra-vascular compartment (Lord 1992). Extrinsic stimuli involving the monocyte during key recruitment events to areas of inflammation include signals derived from adhesion and subsequent transmigration across endothelial cells (Muller & Randolph 1999; Rosenfeld 2002), but also involve chemokine inputs including MCP-1, IL-8 and the CXC chemokine Regulated on Activation Normally T-Expressed presumably Secreted (RANTES) that are relevant to atherosclerosis (Gerszten et al. 1999; Schober et al. 2002). Circulation times for monocytes at early stages of differentiation are between 20-40 hours (Koeffler, Gale, & Golde 1980). If recruited to sites of inflammation following trans-endothelial migration, monocytes may perform phagocytic clearance functions (Duvall, Wyllie, & Morris 1985; Haslett 1992). Alternatively, monocytes may take up tissue specific roles (MacDonald et al. 2002; Massey & Flanagan 1999; Tomita et al. 1994), a phase of monocyte life that is governed by specific cytokine stimuli and may be protracted (Fries, Perneczky, & Kempski 1994).

1.13 Monocyte recruitment and migration

Monocyte behaviour is divided into defined phases from the time of entry into the circulation. Initial chemotactic influences direct intra-vascular monocytes to localise to inflammatory sites such as areas of bacterial infection (Doherty et al. 1987). Further chemotactic stimuli may be provided by complement components thereby increasing cell numbers (Ember et al. 1994). Local cell death at target sites of injury enhances extracellular calcium levels released from apoptotic cells, potentially promoting calcium-dependent monocyte chemotaxis (Olszak et al. 2000) and Fas ligation at macrophage surfaces increases IL-8 and TNF-α production, as well as potentiating neutrophil chemotaxis (Park et al. 2003).

Increasing adhesion molecule expression on endothelial cell surfaces in vessels serving sites of inflammation augments local monocyte recruitment, with increased levels of ICAM-1, E-selectin, and vascular cell adhesion molecule-1 (VCAM-1/CD106) are particularly apparent in immunohistochemical studies of human atheroma (Duplaa et al. 1996).

1.14 Monocyte and endothelial cell interactions

Haemodynamic alterations at individual vulnerable arterial sites, such as branch points, bifurcations, and tortuous curvatures, alter endothelial function. Haemodynamic stimuli in the vessel wall including decreased shear stress and increased turbulence modulate the expression of endothelial adhesion molecules e.g. selectins, VCAM-1 and ICAM-1 (Chappell et al. 1998), which enable more avid binding of monocytes through surface counter-receptors. Molecules mediating trans-endothelial leukocyte migration, such as PECAM-1 (Muller et al. 1993), act in conjunction with chemoattractant molecules, such as MCP-1, osteopontin (Giachelli et al. 1998), and modified LDL to attract monocytes into the artery wall (Rajavashisth et al. 1990).

1.15 Monocyte subsets may differentially modify inflammatory responses

Surface molecular phenotyping has demonstrated that monocyte maturation produces functionally specific phenotypic alterations. Alveolar macrophages express high class II major histocompatibility (MHC) antigens but low CD14; peritoneal macrophages display an inverse phenotype (Andreesen et al. 1990). Plasmacytoid monocytes in interfollicular areas of lymph nodes bear increased levels of CD36+ CD68+ and CD123+ but low levels of CD3, CD11c, CD14, CD20 and CD56 (Facchetti & Vermi 2002), and may progress to dendritic cell morphology and function (Grouard et al. 1997). Kupffer cells in the liver, conversely, are notable for a high surface expression of Mac-2 and Mac-3 (Hashimoto et al. 1996).

Specific subsets of monocytes may be responsible for varying inflammatory responses *in vivo* (Geissmann, Jung, & Littman 2003). The classical phenotype for circulating monocytes is most commonly CD14++/CD16-/HLA-DR+. In disease states with parallel inflammatory cellular mechanisms to atherosclerosis such as rheumatoid arthritis, it has been suggested that CD14+/CD16+ subsets produce significantly different inflammatory effects, as has been demonstrated in clinical studies on rheumatoid arthritis (Kawanaka et al. 2002), systemic sepsis (Skrzeczynska et al. 2002), and pulmonary injury (Horelt et al. 2002; Yoshioka et al. 2002). An alteration in monocyte phenotype with respect to relative CD14 and CD16 (FcγRIII) levels may critically up-regulate TNF-α production responses, thus altering final monocyte/macrophage effector outcomes in inflammation (Belge et al. 2002). The uptake of differentially modified lipids also appears to be heterogenous in monocyte populations:

CD14+/CD16+ subsets display higher uptake of enzymatically degraded LDL in contrast to naïve CD14++/CD16- subsets, resulting in rapid ultra-structural transformation of CD14+/CD16+ monocytes into foam cells (Kapinsky et al. 2001). Acetylated, native or even copper-oxidised LDL uptake is less avid, with little involvement of classical scavenger receptor pathways including SR-A and CD36.

Associations have also been made between hyperlipidaemia and rare monocyte subsets, with HDL levels inversely correlating to the population size of CD64-CD16+ monocytes. Subjects with high levels of particular lipoproteins, in particular apolipoprotein E3/E4 and E4/E4 phenotypes, show a tendency towards larger circulating populations of CD64-CD16+ monocytes (Rothe et al. 1996). Such pre-differentiated monocyte subtypes may specifically target the vascular wall and at least three different types of macrophages, each regulated by different T-cell cytokines have been identified (Tormey et al. 1997). Differences in arterial endothelium and micro-vascular endothelium may exist to preferentially attract different types of monocytes (Garlanda & Dejana 1997). If individual monocyte inflammatory responses can specify lipid handling and modification during the transition from monocyte into foam cell, these studies raise the possibility that individual monocyte subsets and differentiated monocytes play varying roles in atherogenesis and foam cell formation.

Auto-immune processes may be driven by a change in monocytes towards a dendritic cell phenotype, potentially involving immune presentation of epitopes within the plaque in a way that prolongs inflammation. Monocyte sub-differentiation into dendritic cells has been linked to a number of microenvironmental stimuli including critical adhesion interactions and movement of monocytes across endothelial monolayers. Interactions mediated by CD31 or PECAM (Muller & Randolph 1999) may be implicated in the maturation of monocytes entering lymph nodes, and subsequent transformation into dendritic cells. Influences upon monocyte differentiation by pro-atherogenic stimuli are thus relevant to innate immune and adaptive immune responses.

1.16 Monocyte maturation and foam cell differentiation

Previously described by light microscopic appearances, the presence of foam cells has been documented as a basic ultrastructural feature of atheroma (Buja, Kovanen, & Bilheimer 1979), but it is only in the recent past that attempts have been made to address the origins of such cells (Gerrity 1981a), and explain the cellular dynamics of these lesions (Jerome & Lewis 1997). Immunophenotyping originally suggested that foam cells were derived from monocytes, rather than being lipid laden smooth muscle cells (Aqel et al. 1984). Once resident within plaque these monocyte-derived foam cells still maintain certain phenotypic

and functional features of monocyte/macrophages (Inaba et al. 1993; Trogan et al. 2002), suggesting that a dynamic inflammatory role for this cell population might be fundamentally important in plaque behaviour. Data from experiments involving the *in vitro* exposure of monocytes to lipoproteins, and to other pro-inflammatory and pro-atherogenic stimuli have suggested that the local environment regulates the progression of naïve monocytes towards a foam cell phenotype (Wang et al. 1996). Alteration of this phenotype may determine monocyte functional responses within an atheromatous lesion, with the production of extracellular matrix metalloproteinase inducer (EMMPRIN) (Major et al. 2002). Ox-LDL, TNF-α, or M-CSF all cause concentration and time-dependent increases in macrophage production of MMP-3 (Uzui et al. 2002) contributing to proteolytic degradation of extracellular matrix. This model of inflammatory plaque destabilisation offers potential for future therapies, as an ability to modify plaque inflammation may limit further injury, and promote vascular repair.

1.17 Monocyte apoptosis

Although the mechanisms of apoptotic cell clearance by monocyte-derived macrophages have been extensively researched (Henson, Bratton, & Fadok 2001), relatively less attention has been paid to death programmes in monocytes than in granulocytes. However, apoptosis in monocyte pre-cursors perform a physiologically important role in haematopoiesis (Ohishi et al. 2000), and peripheral blood monocytes may undergo programmed cell death in common with other leukocytes (Mangan & Wahl 1991). Monocyte susceptibility to apoptosis varies with maturation status (Kiener et al. 1997), the response to extrinsic stimuli including lipopolysaccharide (LPS) exposure (Um, Orenstein, & Wahl 1996), and the influence of cytokines including IL-4 (Mangan, Robertson, & Wahl 1992). Active sites of inflammation may provide an environment that is non-supportive of uncommitted immature monocyte survival. In atherosclerotic plaque, the absence of non-cytokine protective serum components such as lysophosphatidic acid (Koh et al. 1998) may expose the naïve monocyte to pro-apoptotic signals, and engagement of apoptotic pathways may be accelerated. Leukocyte apoptosis may be induced by discrete cellular triggers, including the surface ligation of CD95 (Fas) (Iwai et al. 1994), and the withdrawal of survival proteins including key cytokines such as macrophage colony stimulating factor (Munn et al. 1995). Monocyte apoptotic behaviour contrasts with that seen in granulocytes, which display induction of cell death *via* TNF-α ligation of the TNF-R1 receptor (Murray et al. 1997), driving death *via* TRADD (TNF receptor associated death domain) (Hsu, Xiong, & Goeddel 1995), FADD (Fas associating protein with death domain), and RAIDD (receptor interactivity protein

(RIP)-associated ICH-1/CED-3 homologous protein with a death domain). Monocytes in distinction appear to be activated by TNF ligation (Langstein et al. 1998), and TRAIL (TNF-related apoptosis-inducing ligand) which confer protection against cell death *via* activation of NF-κB (Secchiero et al. 2003). The pro-apoptotic cytokine TRAIL (TNF-related apoptosis-inducing ligand) acts to promote both apoptosis and cell activation, performing a complex dual role analogous to that of TNF receptors (Schneider et al. 1997; Walczak et al. 1997). TWEAK/Apo3L, another TNF family member (Chicheportiche et al. 1997), acts to initiate the downstream activation of caspases (Nakayama et al. 2002) and can cause monocyte apoptosis that may be important in auto-immune disease (Kaplan et al. 2002). Caspases, cysteine proteases that have been highly conserved through evolution, activate nucleases including caspase-activated DNAse (Thornberry & Lazebnik 1998). The resultant cleavage of DNA causes nuclear fragmentation, one of the hallmarks of apoptosis, which has been demonstrated in spontaneous monocyte apoptosis (Fahy, Doseff, & Wewers 1999). TWEAK is expressed on human monocytes at low levels, but appears to be upregulated following interferon gamma exposure, adding a further level of sophistication to monocyte apoptotic control mechanisms in the context of inflammation (Nakayama et al. 2000). Phagocyte uptake of apoptotic cells is regulated by integrin mediated signalling, and dependent upon the number of apoptotic cells already ingested (Erwig et al. 1999). Phagocytosis of apoptotic cells may alter inflammatory balance, promoting TGFβ production (Huynh, Fadok, & Henson 2002) and cross-presenting ingested antigens (Savill et al. 2002), events discussed further in Chapters 3 and 6. Unregulated apoptosis *in vivo*, especially in the context of overburdened or inefficient clearance mechanisms, may lead to marked cellular necrosis (Ogasawara et al. 1993) increasing local pro-inflammatory mediators levels and exacerbating local cell damage. This may be a contributory factor in the protracted pro-inflammatory state present within unstable plaque. Balanced apoptotic cell clearance is thus of central importance in maintaining plaque dynamics in a stable pattern.

1.18 Growth factor influences upon monocytes and atherogenesis

Growth factors regulate vascular repair and neovascularisation processes relevant to arterial disease. Vascular endothelial growth factor (VEGF) promotes angiogenesis and may also regulate the rate of atherosclerotic plaque development. Data from both rabbit models and murine apolipoprotein E/apolipoprotein B100 gene deletion models suggest that VEGF increases atherosclerotic plaque area, as well as plaque macrophage and endothelial cell content (Celletti et al. 2001). Ox-LDL increases VEGF mRNA and protein expression in the monocytic cell line RAW 264. Immunohistochemical analyses of human atherosclerotic

plaques demonstrate VEGF staining in foam cell-rich regions of plaque adjacent to the lipid core and neovascularized basal regions, with VEGF localising to macrophage cell surfaces (Ramos et al. 1998). Monocytes entering atherosclerotic plaque thus potentiate neovascularisation, with increased visible capillary density and tissue perfusion seen in murine models (Silvestre et al. 2003). Neovascularisation at a microscopic level may provide alternative routes for leucocytes recruitment into lesions (Moulton et al. 2003). Microvessel density in lipid-rich plaques is higher than fibrous plaques, with most vessels located in shoulder-regions or the base of the plaque, adjacent to intra-plaque inflammatory cellular infiltrates. Microvessels in lipid-rich plaques show increased levels of ICAM-1, VCAM-1, E-Selectin and CD40, with VEGF being confined to vessels and mononuclear cells within lipid-rich plaques alone. Increased cellular influx may augment local inflammation, potentially increasing plaque vulnerability (de Boer et al. 1999). The sequestration, survival, and replication of monocytes in atheromatous plaque are partially dependent upon M-CSF and granulocyte-macrophage colony-stimulating factor (GM-CSF). Prolonged exposure to M-CSF has been reported to rescue macrophages from cell death *in vitro* and, within the lesions, may offer a mechanism for local monocyte/macrophage replication and proliferation. In contrast, inflammatory cytokines such as interferon-γ (IFNγ) activate macrophages and under certain circumstances induce them to undergo apoptosis.

1.19 PPARγ: a role in monocyte differentiation, survival and functional regulation

Nuclear receptors are involved in regulation of leukocyte cellular homeostasis and inflammatory responses, and have numerous actions in metabolic control and cellular survival. Nuclear receptors are ligand-activated transcription factors which mediate steroid hormone, thyroid hormone, and fat-soluble vitamin signaling (Evans 1988). Orphan nuclear receptors, with initially unknown ligands, target genes and physiological functions, bear homology to steroid nuclear hormone receptors. The role of the peroxisome proliferator receptor gamma (PPARγ), previously regarded as an orphan nuclear receptor is increasingly studied with regard to glucose homeostasis, adipocyte metabolism and leukocyte inflammatory function (Giguere 1999). All nuclear receptors have a DNA binding site and a ligand binding site, enabling the receptor to bind to specific hormone response elements within the gene (Chambon 1996). PPARγ acts *via* heterodimerisation with retinoid X receptor (RXR) ligands, to bind a PPAR response element (PPRE) within the genome thus regulating transcription of target genes (Tugwood et al. 1992). PPARγ appears to alter cellular phenotype and function dependent on cell type and cell environment which has

relevance to atherogenic processes (Chinetti, Fruchart, & Staels 2001; Ricote et al. 1999). Variation in responses may be partially attributable to differential expression of PPAR subtypes as well as cell specificity, dictating differential transcription for monocyte/macrophages in distinction to adipocytes (Spiegelman 1998) hepatocytes (Schoonjans et al. 1996) or endothelial cells (Marx et al. 1999).

1.20 PPARγ effects on lipid handling and phenotype

PPARγ induction of an altered monocyte surface phenotype increases lipid entry into the cell *via* the scavenger receptor B, CD36 (Huang et al. 1999a; Tontonoz et al. 1998) but downregulates lipid influx *via* other routes such as scavenger receptor A (SR-A) (Ricote et al. 1998b). Initially it was suggested that *in vitro* PPARγ ligation by specific ox-LDL derivatives produced massive lipid accumulation and the production of a foam cell phenotype (Tontonoz et al. 1998). However, net lipid balance is mitigated by lipid efflux *via* the ABC1A transport mechanism and is also under the control of PPARγ and the liver X receptor (LXR) (Chawla et al. 2001b), which may produce a net lipid deficit dependent upon the predominant lipid type present. Scavenger receptor expression is controlled during inflammatory responses, *via* TGF-β_1 and TGF-β_2 phosphorylation of MAP kinase. Subsequent MAP kinase phosphorylation of PPARγ in turn reduces CD36 gene transcription (Han et al. 2000). Thus, altered lipid handling due to ligation of PPARγ does not result necessarily result in acquisition of a foam cell phenotype, but may have more subtle effects upon monocyte/macrophage function.

1.21 PPARγ and inflammation

Inflammatory mediators are implicated in PPARγ ligation and subsequent transcriptional regulation of inflammatory cells. The cyclopentenone prostaglandins 15dPGJ$_2$ and Δ12PGJ$_2$, products of arachidonic acid metabolism derived from the hydroxylation of Prostaglandin D$_2$ to Prostaglandin J$_2$, are present in normal inflammatory reactions and have been noted to act as natural ligands for PPARγ (Kliewer et al. 1995). They have been demonstrated to govern survival in leukocytes (Chinetti et al. 1998) and endothelial cells (Bishop-Bailey & Hla 1999) and act as anti-inflammatory modulators of macrophage function aiding the resolution of inflammatory injury (Ricote et al. 1998b). Furthermore, given the role of glucose regulation by PPARγ, specific assessment of the role of PPARγ in monocyte microenvironmental responses is important, as diabetic disease correlates strongly to clinical atherosclerotic progression (Glass 2001). Thus, because cyclopentenones may modulate

inflammation and cell survival within atherosclerotic plaque, a potential link to monocyte apoptosis through PPARγ will be explored in this thesis.

1.22 Monocytes and innate immune responses

Peripheral blood-derived monocytes, the precursor of tissue macrophages, are present at all stages of atherogenesis. Monocyte-derived macrophages act to scavenge cellular debris and perform as antigen-presentation cells in concert with both B and T lymphocytes. The role of monocytes in innate immune mechanisms is crucial and inflammatory injury outcomes rest on the balance of their functions. They are responsible for the local secretion of cytokines, chemokines, growth-regulating molecules, metalloproteinases and other hydrolytic enzymes, thus regulating potential extracellular matrix degradation. Altered chemotaxis and increased accumulation of monocytes in fatty streaks may be mediated by differentially regulated chemokine expression (Boisvert et al. 1998; Boring et al. 1997). Monocyte activation results in increased surface expression of chemokine receptors, mucin-like molecules that bind selectins, integrins that bind adhesion molecules in the immunoglobulin superfamily, and receptors that bind chemoattractant molecules (Springer TA & Cybulsky MI 1996) (see Chapter 6 section 6.1.6). These classical inflammatory ligand–receptor interactions further activate mononuclear cells, induce cell proliferation, promoting and localizing inflammatory responses at lesion sites. In a number of inflammatory diseases, elevated plasma levels of cleaved plasma membrane adhesion receptors have been reported, and taken to be markers of a sustained inflammatory response. Metalloproteinase activity results in the cleavage of L-selectin (by L-selectin sheddase) and the cleavage of CD18. Some cell-surface molecular shedding may be undertaken by disintegrins, sometimes called metalloproteinase-like, disintegrin-like, cysteine-rich proteins (MDCs) or a disintegrin and metalloproteinase (ADAMs), identifiable in endothelium, smooth muscle, and macrophages. These trans-membrane proteins contain a metalloproteinase sequence in their extracellular segment permitting activation of molecules such as TNF-α (Black et al. 1997; Moss et al. 1997). Although absent in normal arteries, one particular disintegrin, MDC15/ADAM15, has been shown to be present in lesions of atherosclerosis (Herren, Raines, & Ross 1997). Increased plasma concentrations of cleaved cell surface molecules might thus be used to identify patients at risk for atherosclerosis or other inflammatory diseases.

Although smooth muscle cells were the only cells thought to proliferate during expansion of atherosclerotic lesions, monocyte-derived macrophage replication is now thought to be equally important in plaque progression. The ability of macrophages to produce cytokines including TNF-α, IL-1, and TGF-ß, proteolytic enzymes including metalloproteinases, and

growth factors such as PDGF and insulin-like growth factor (IGF)-I, may be critical for the cycles of damage and repair that ensue as the lesions progress. Thus resident plaque monocyte/macrophages may dictate plaque behaviour and hence clinical outcomes more than other resident cell types including smooth muscle cells (Rosenfeld & Ross 1990).

Since activated macrophages express class II histocompatibility antigens and have the potential to present antigen to CD4 and CD8 T lymphocytes present within developing lesions, (Raines EW, Rosenfeld ME, & Ross R 1996) cell-mediated immune responses may also be involved in atherogenesis (Hansson et al. 1989). T cells are activated when they bind antigen processed and presented by macrophages in the context of co-stimulatory molecules such as CD80, CD86 and CD40 Ligand. T-cell activation results in the secretion of cytokines, including interferon-γ and TNF-α and ß, amplifying the inflammatory response. Lesional smooth-muscle cells that express Class II molecules may also present antigens to T cells. One putative antigen may be ox-LDL, (Stemme et al. 1995) which can be produced by macrophages (Folcik, Aamir, & Cathcart 1997). Heat-shock protein 60 may also contribute to autoimmunity. This and other heat-shock proteins perform several functions, including the assembly, intracellular transport, and breakdown of proteins and the prevention of protein denaturation. Heat shock proteins may be present at elevated levels on endothelial cells and provide a stimulus for innate immune responses thus contributing to local arterial wall inflammation (Xu et al. 1993).

1.23 Animal models of atherosclerosis

The use of targeted gene deletions has increased our understanding of the role played by individual components of the leukocyte inflammatory response in atherogenesis particularly animals bearing gene deletions for apolipoprotein E (apo E) (Nakashima et al. 1994; Plump et al. 1992) and the LDL receptor (LDL-R)(Kowala et al. 2000), that mimic elevated plasma lipoprotein states seen in human subjects. Recent work using double knockouts of apo E and LDL-R (Ishibashi et al. 1994), shows aggressive atherosclerosis in these animals more representative of human atherosclerotic lesions (McGillicuddy, Carrier, & Weinberg 2001). In the absence of apolipoprotein E, lipoprotein remnants are not carried to the liver, where they are normally metabolized, and the mice become hypercholesterolaemic and lesions of atherosclerosis develop that are similar to those in humans. Murine models emphasise the importance of adhesion molecules in monocyte recruitment to atherosclerotic plaque, and subsequent monocyte phenotypic change. Mice deficient in apolipoprotein E, show increased ICAM-1 expression at lesion-prone sites, above baseline levels seen in wild-type animals. In contrast, VCAM-1 is absent in normal mice but is is also expressed at lesion-prone sites in

mice with apolipoprotein E deficiency (Nakashima et al. 1998). Thus, adherence of monocytes may be driven by increased expression of one or more of the different families of adhesion molecules, induced primarily by increased local lipid levels. Blockade of adhesion molecules might thus be predicted to be protective. In mice deficient in intercellular adhesion molecule 1, P-selectin, CD18, or combinations of these molecules, lipid supplementation was shown to cause smaller atherosclerotic lesions (Hynes & Wagner 1996). The work presented here does not make use of murine models, focusing instead on responses of primary human cells. However, it was felt to be important to address altered monocyte transcription of adhesion molecules, following exposure to varying forms of LDL. This, in conjunction with surface molecular phenotyping, may offer insights into the changes occurring in the transition from uncommitted circulating monocyte to a mature plaque-based macrophage with an altered phenotype.

Section E Hypothesis and specific aims of the work

The overall hypothesis being proposed is that pro-atherogenic and pro-inflammatory microenvironments affect monocyte survival and monocyte phenotype.

1.24 Specific aims and structure of thesis

The specific aims of this work are outlined.

a) To determine whether modified lipoproteins and inflammatory mediators alter monocyte survival, the effects of both LDL and cyclopentenone prostaglandins upon monocyte apoptosis will be assessed in Chapter 3.

b) To evaluate the role of PPARγ in monocyte survival the effects of PPARγ ligands upon monocyte apoptosis will be assessed, and intra-cellular levels of monocyte PPARγ will be measured in Chapter 3.

c) The dependence of monocyte apoptosis upon PPARγ will be specifically explored using a dominant co-repressor mutant gene to suppress PPARγ gene function, and will be described in Chapter 4.

d) Changes in monocyte transcription following LDL exposure will be evaluated using gene miniarrays in Chapter 5.

e) To determine whether modified lipoproteins and inflammatory mediators alter monocyte phenotype, the effects of LDL upon monocyte structure, and of both LDL and cyclopentenone prostaglandins upon monocyte surface molecule expression, will be assessed in Chapter 6.

f) The data presented will be set in the context of current literature, with a view to potential further work and clinical relevance in Chapter 7.

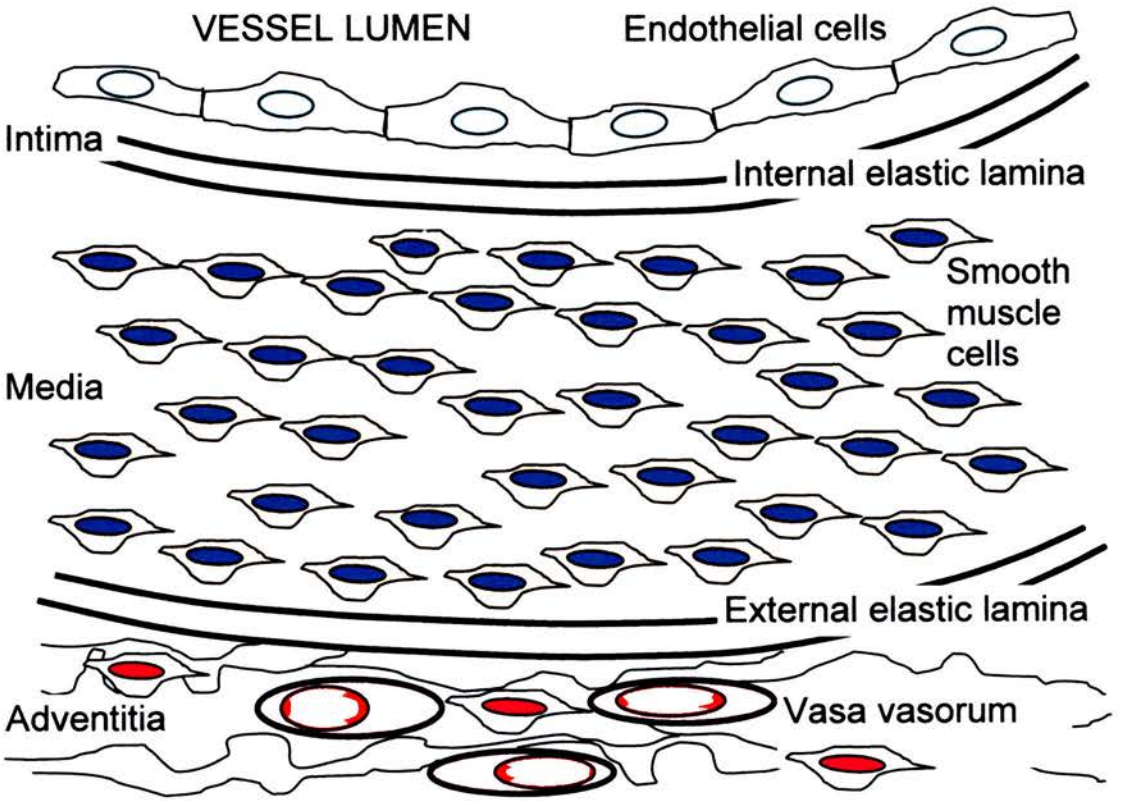


Figure 1-1 Normal artery wall structure

Blood in the vessel lumen is separated from the artery wall and thrombogenic matrix proteins by the endothelial monolayer.

The intima is bounded by the internal elastic lamina, separating this sub-endothelial area from the smooth muscle rich media. Further structural strength is offered by the external elastic lamina.

The adventitia is rich in matrix proteins and bears vasa vasora, lymphatic channels and nerve endings, in addition to smooth muscle cells.

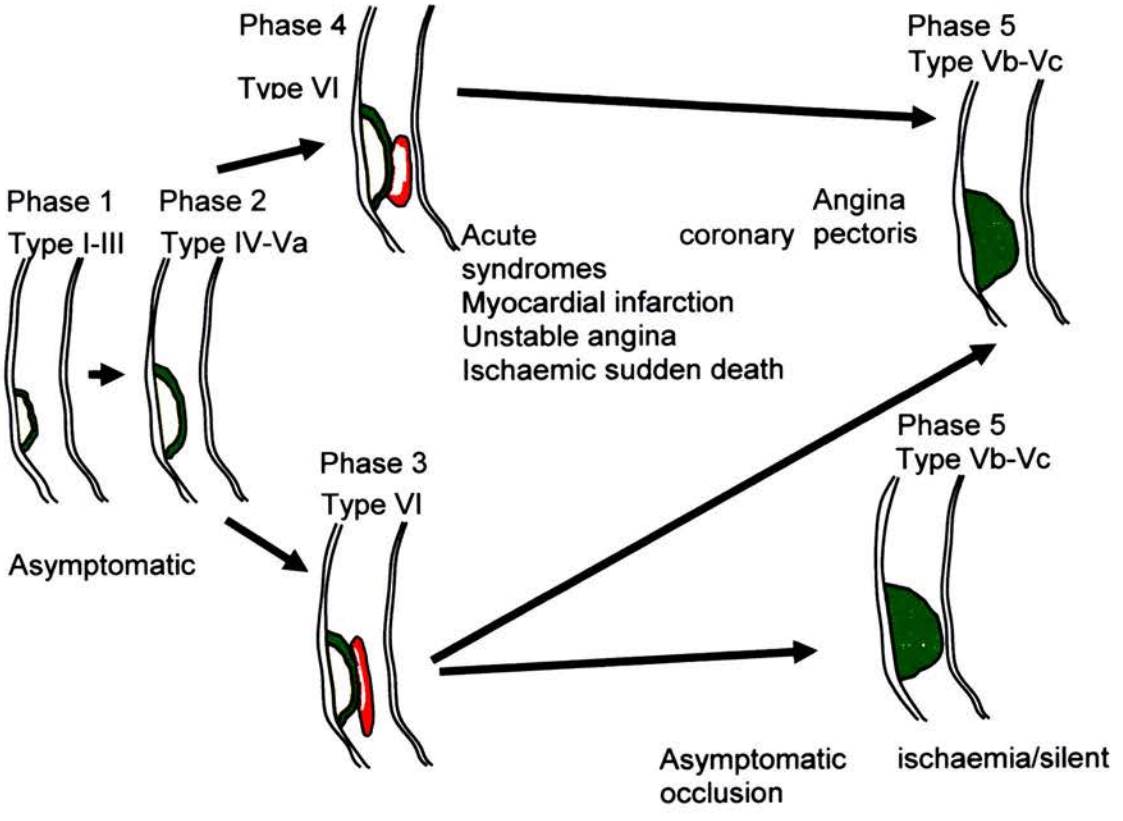


Figure 1-2 American Heart Association classification of atherosclerosis

Initial pre-atheromatous lesions (fatty streaks) graded type I-III are clinically silent and may not progress to produce symptoms (Phase 1). More advanced atherosclerotic lesions may also be clinically silent (type IV and Va lesions, clinical Phase 2).

Increasing lesion complexity results in changes in phase of disease, and eventually leads to the involvement of thrombus within a Type VI lesion. If this is clinically silent (Phase 3), it may resolve leaving an organised type V lesion. This again may be clinically silent or produce symptoms of chronic ischaemia (Phase 5).

Alternatively a Type VI thrombotically complicated lesion may progress rapidly to cause vessel occlusion and potentially fatal outcomes (phase 4). Resolution of such a thrombotic episode is possible, resulting in a stable Type V lesion that is often associated with clinical symptoms (Phase 5).

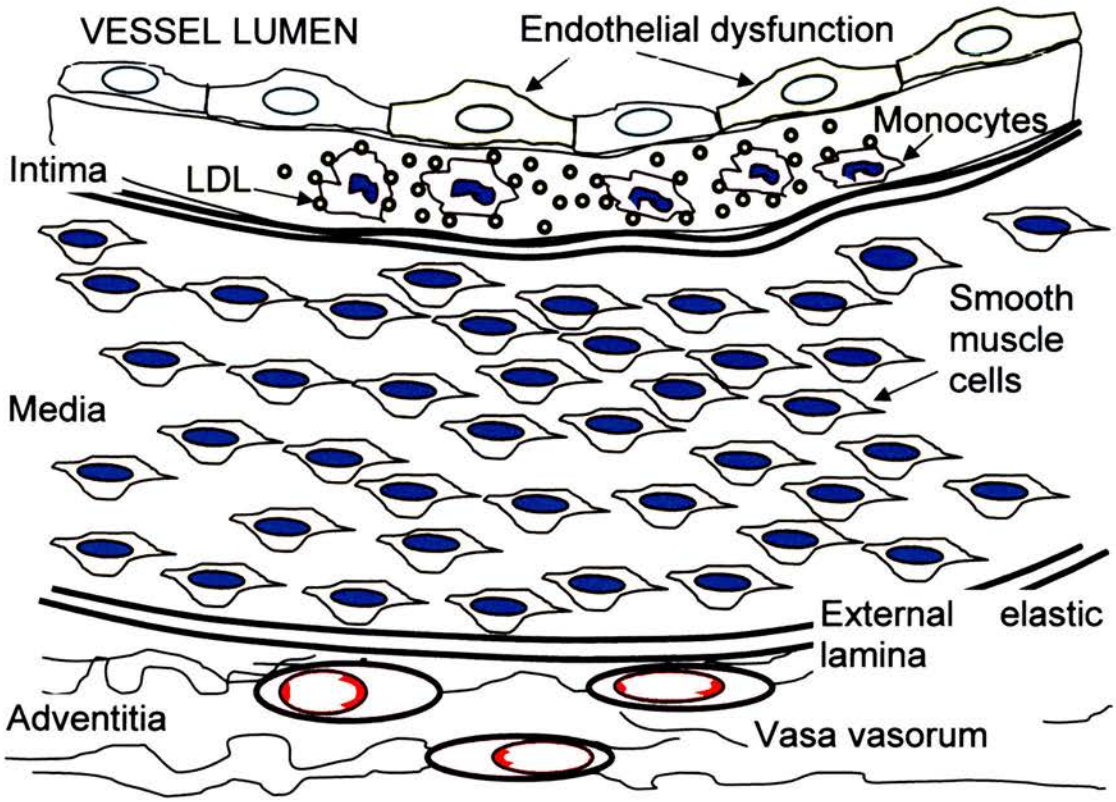


Figure 1-3 Gross overview of fatty streak formation

Lipid retention, denoted by small yellow spheres in the sub-endothelial space, alters the intima, and enhances leukocyte infiltration. Endothelial dysfunction may enhance lipid penetration of the artery wall. A predominant influx of monocytes is seen in this early stage of atherogenesis, with preservation of medial and adventitial structure. This corresponds to the early clinically silent Phase 1 lesions in the Stary/American Heart Association (AHA) classification, with lesions from stage I to III.

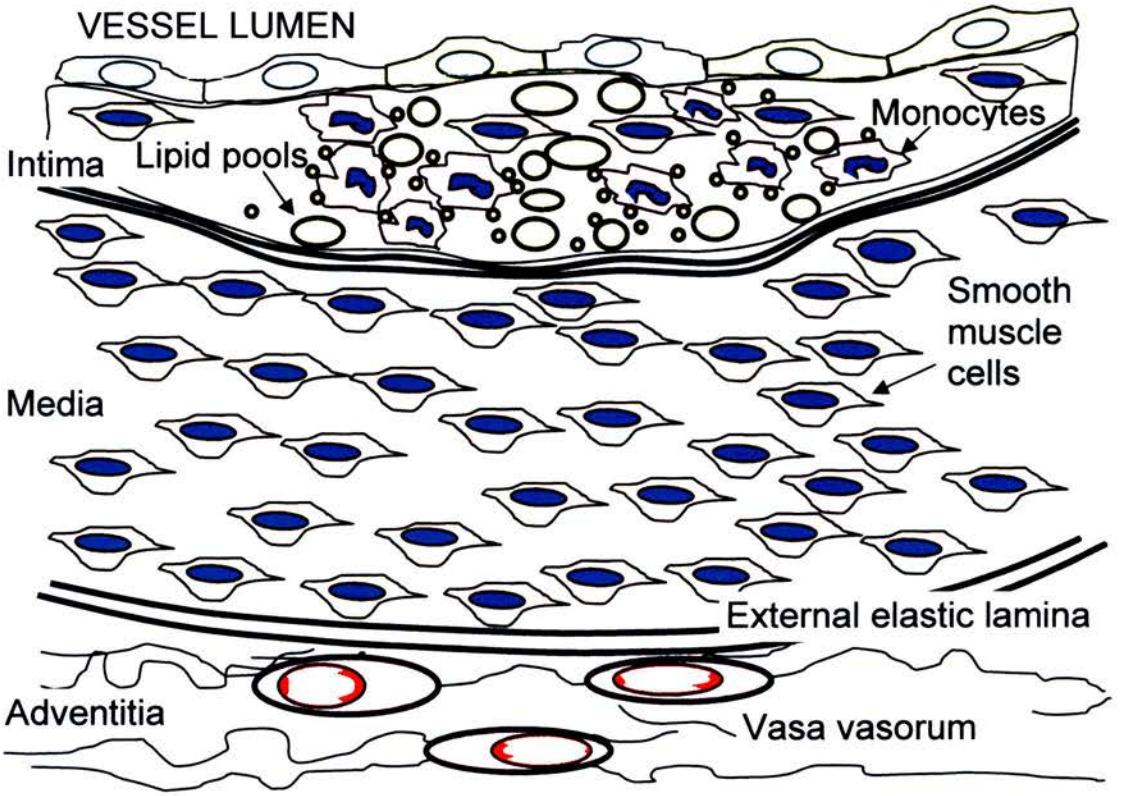


Figure 1-4 Early atheroma

Increasing macrophage infiltration into the intima is accompanied by the formation of extracellular lipid pools. Smooth muscle cells and T cells are present in the intima which appears diffusely thickened, with an increase in connective tissue.

These AHA Type IV lesions are termed atheroma, due to their gruel-like composition.

Although clinically silent (Phase 2), they may progress to plaque erosion, rupture or thrombosis (Phase 3 or 4).

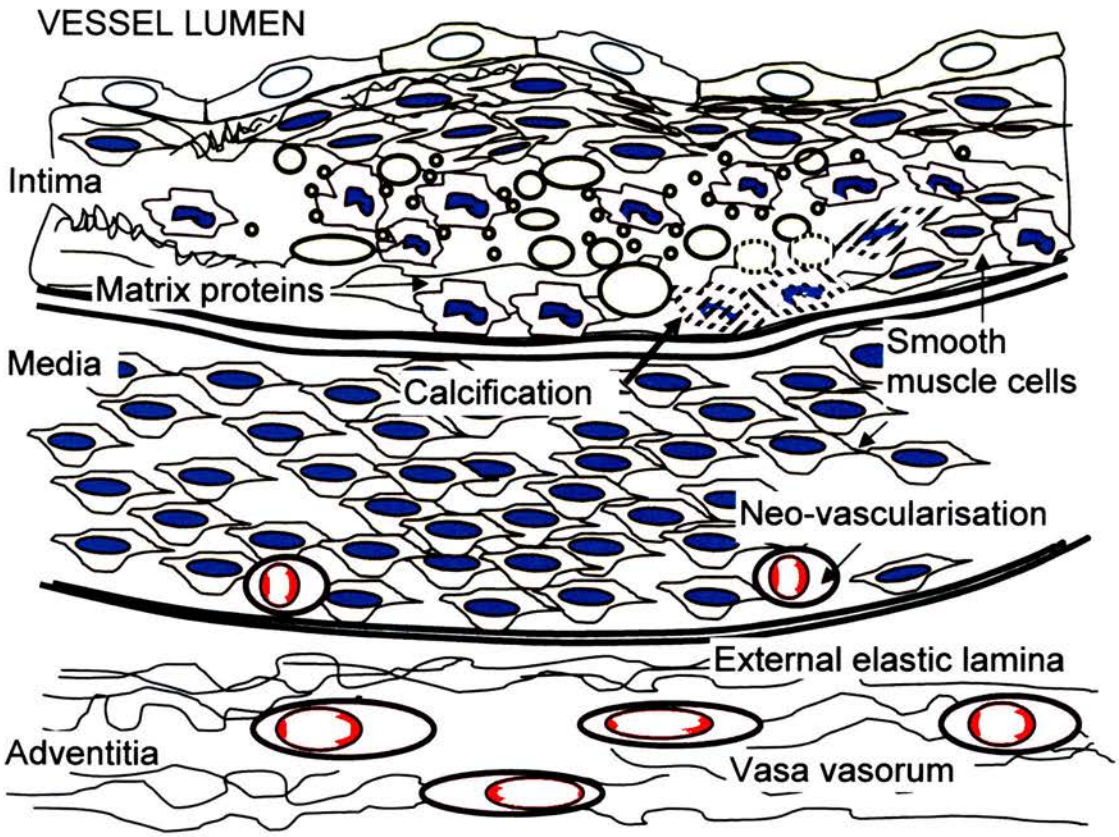


Figure 1-5 Advanced atheroma

Smooth muscle infiltration into the intima along with matrix protein synthesis promotes fibrous cap formation sub-endothelially, adding to lesion bulk.

Further increases in macrophage populations are seen, and neo-vascularisation is visible in the media as well as adventitia.

These AHA type V lesions are also known as fibro-atheroma, and may be complicated by thrombus formation at the luminal surface.
Type Va lesions have multiple lipid cores, and are usually clinically silent (Phase 2), but may progress to plaque rupture.
Type Vb lesions have calcified contents, and type Vc lesions may have reduced or minimal lipid content.

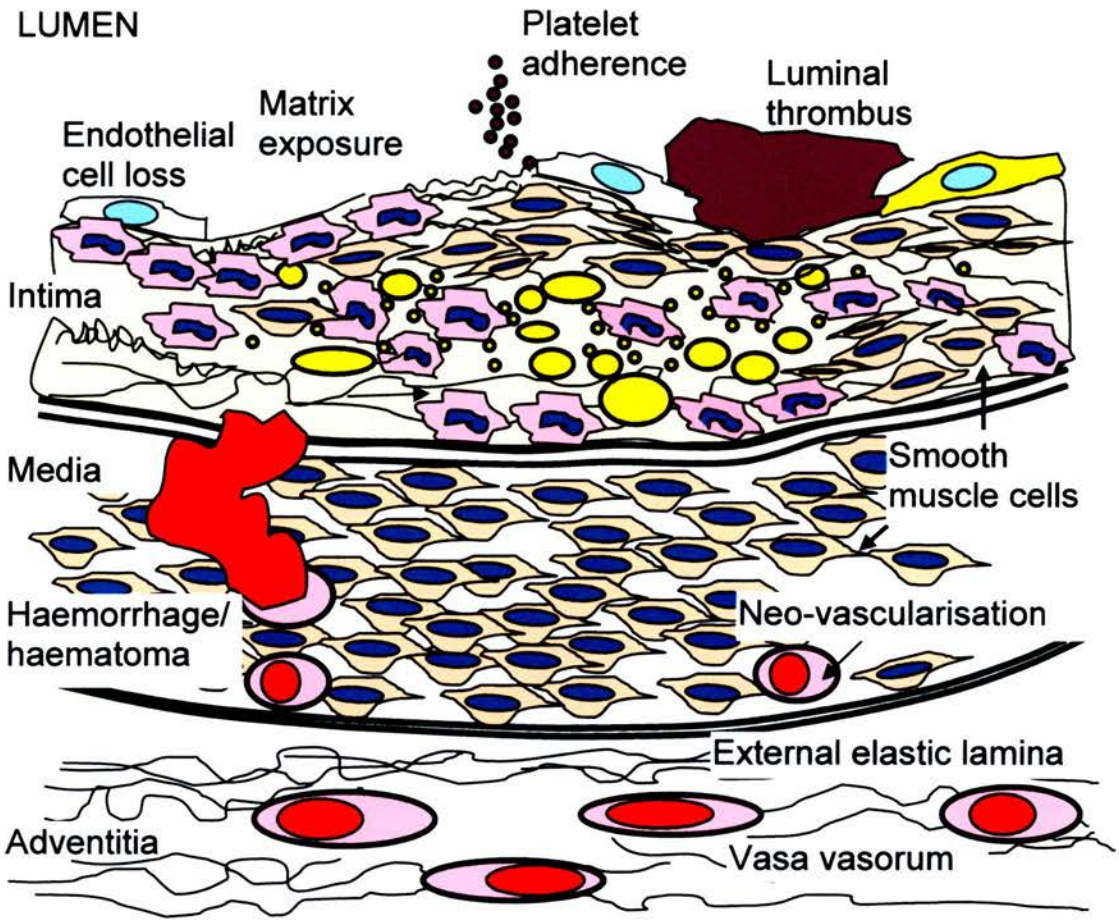


Figure 1-6 Complex atheroma

Progression of advanced plaque to clinically complex lesions may be driven by endothelial cell loss and/or plaque fissuring, often at areas of high macrophage density, causing progression to AHA type VI lesions.

Surface defects have been classed as type VIa lesions and may represent rapid progression form type IV or type Va lesions.

Intra-plaque haemorrhage may contribute to lesion volume, in type VIb lesions. If this extends into the more luminal areas of plaque, this may potentially disrupt plaque architecture.

Exposure of matrix components to intra-luminal blood initiates platelet adherence, causing local thrombus formation in AHA type VIc lesions. Luminal thrombus may resolve, and be incorporated into the plaque adding to lesion bulk.

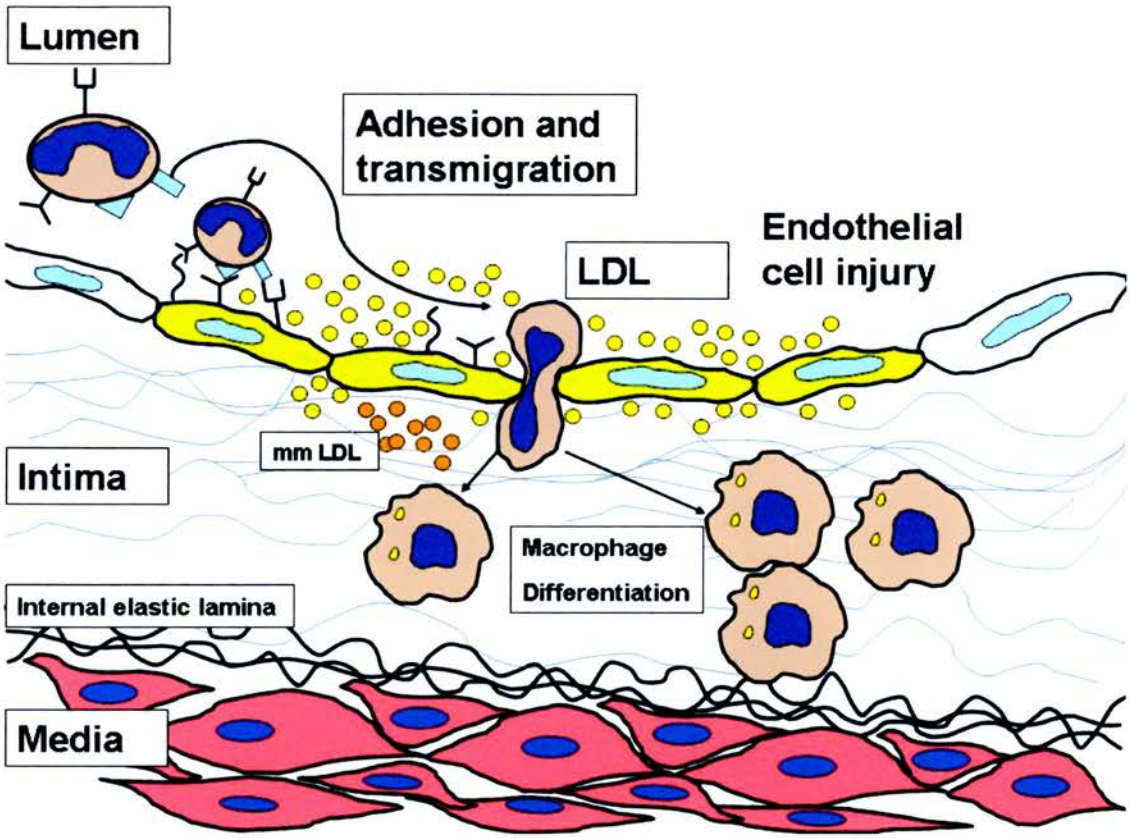


Figure 1-7 Early inflammatory vessel wall injury in atherosclerosis

Vascular injury mediated by factors including LDL, hypertension, shear stress and glycosylation end products may induce endothelial dysfunction (denoted by green endothelial cells), accompanied by a reduction in local nitric oxide production.

Systemic inflammation during microbial infection may contribute to vascular inflammation and endothelial dysfunction, although this remains controversial.

Associated increases in adhesion molecule expression direct leukocyte transmigration into the vessel wall. The influx of monocytes is most notable, although a role for granulocytes in early atherogenesis is possible.

Early leukocyte lipid and collagen deposition correspond to fatty streaks, or Stage I in the Stary/American Heart Association classification of atheromatous lesions.

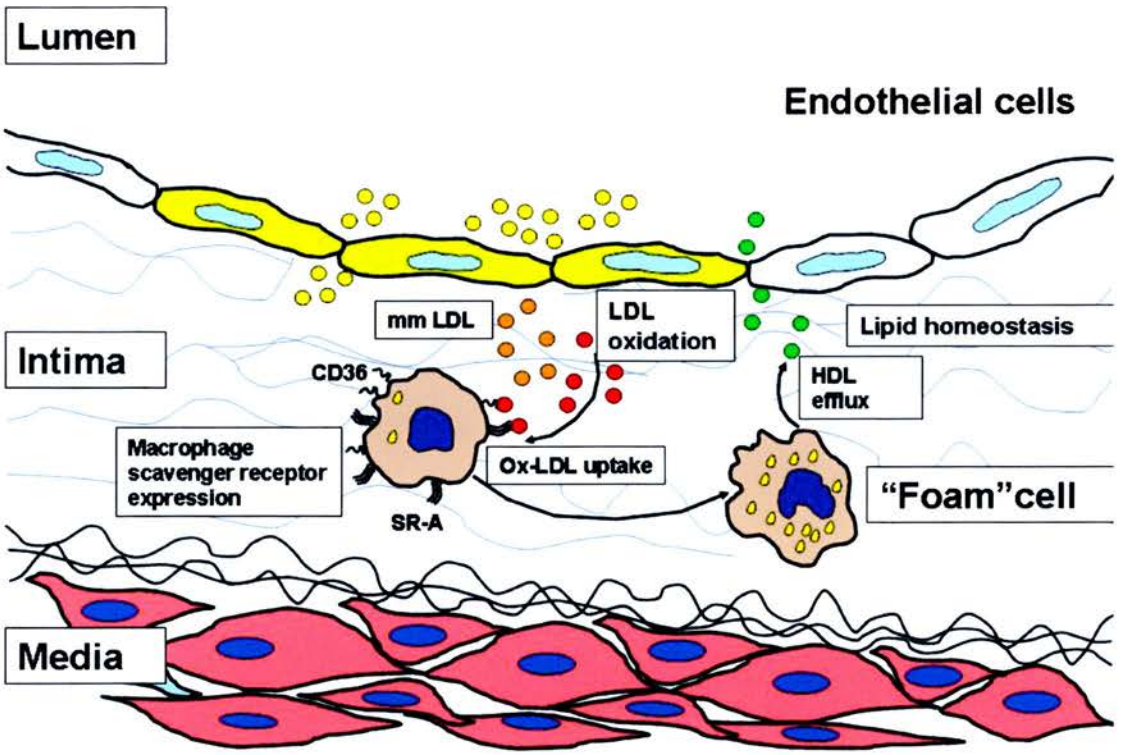


Figure 1-8 Inflammatory contributions to fatty streak formation

Monocytes exit the lumen and differentiate according to local cellular stimuli including cytokines and inflammatory mediators.

LDL is subject to progressive oxidative changes in the sub-endothelial space. Both minimally modified LDL (mmLDL) and oxidised LDL (ox-LDL) contribute to macrophage lipid uptake via macrophage scavenger receptors. Scavenger receptors are a large family of surface molecules, but key players in macrophage uptake of altered LDL appear to be scavenger receptor A (SR-A) and scavenger receptor B-I or CD36.
Cholesterol efflux mechanisms act to limit lipid accumulation, and drive HDL efflux via the ABCA1 transporter mechanism, a homeostatic process that appears to be under the regulation of nuclear receptors, including PPARγ. However, excessive local lipid concentrations may overwhelm the capacity of these lipid disposal routes.

This level of macrophage infiltration corresponds to early changes seen in type II Stary/AHA lesions.

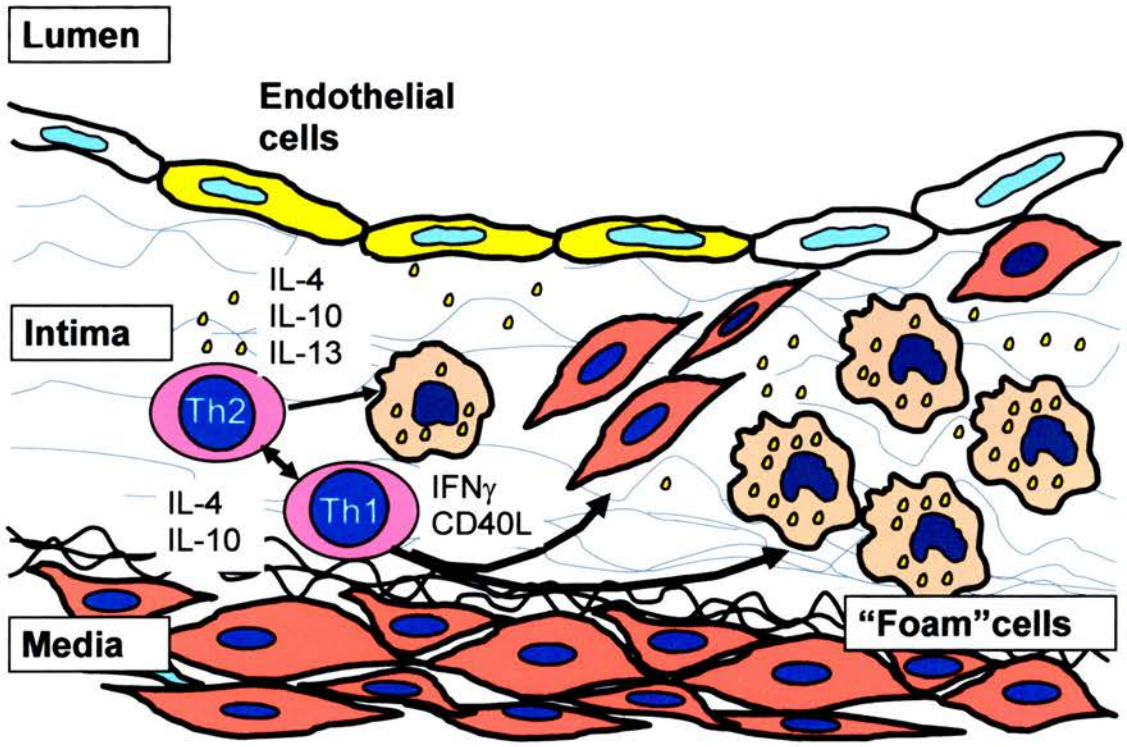


Figure 1-9 Immune mechanisms promote atherosclerotic progression

The influx of T-lymphocytes promotes a chronic inflammatory response in the vessel wall. Cytokines, derived from pro-inflammatory T-helper1 (Th1) and anti-inflammatory T-helper2 (Th2) cells, regulates macrophage and vascular smooth muscle cell (VSMC) responses in atherosclerosis.

Th1 inputs, including the signalling molecule CD40 ligand (CD40L), influence VSMC migration into the intima from the arterial media. Lesion bulk is further enhanced by fibroblastic VSMC responses including collagen synthesis.
However, Th1 cells also stimulate both macrophages and VSMCs with interferon gamma (IFNγ), to promote macrophage Class II major histocompatibility (MHC) expression, decrease collagen synthesis and inhibit VSMC proliferation.
Th1 signalling via the CD40/CD40L dyad to macrophages enhances secretion of matrix metalloproteinases (MMPs), contributing to extracellular matrix degradation in the shoulder areas of plaque. Th1 CD40 signalling to VSMCs also enhances tissue factor production, promoting local thrombus formation.

The Th2 derived cytokines interleukin (IL)-4, IL-10 and IL-13 limit Th1 activity, antagonise IFNγ, and reduce pro-inflammatory macrophage function.

Th1 and Th2 cells self-regulate their responses via IL-4 and IL-10 feedback.
T cells may also present ox-LDL derived antigens, suggesting an adaptive immune component to atherosclerotic disease.

These lesions correspond to the changes in Stary/AHA stage III and early stage IV atherosclerotic plaque.

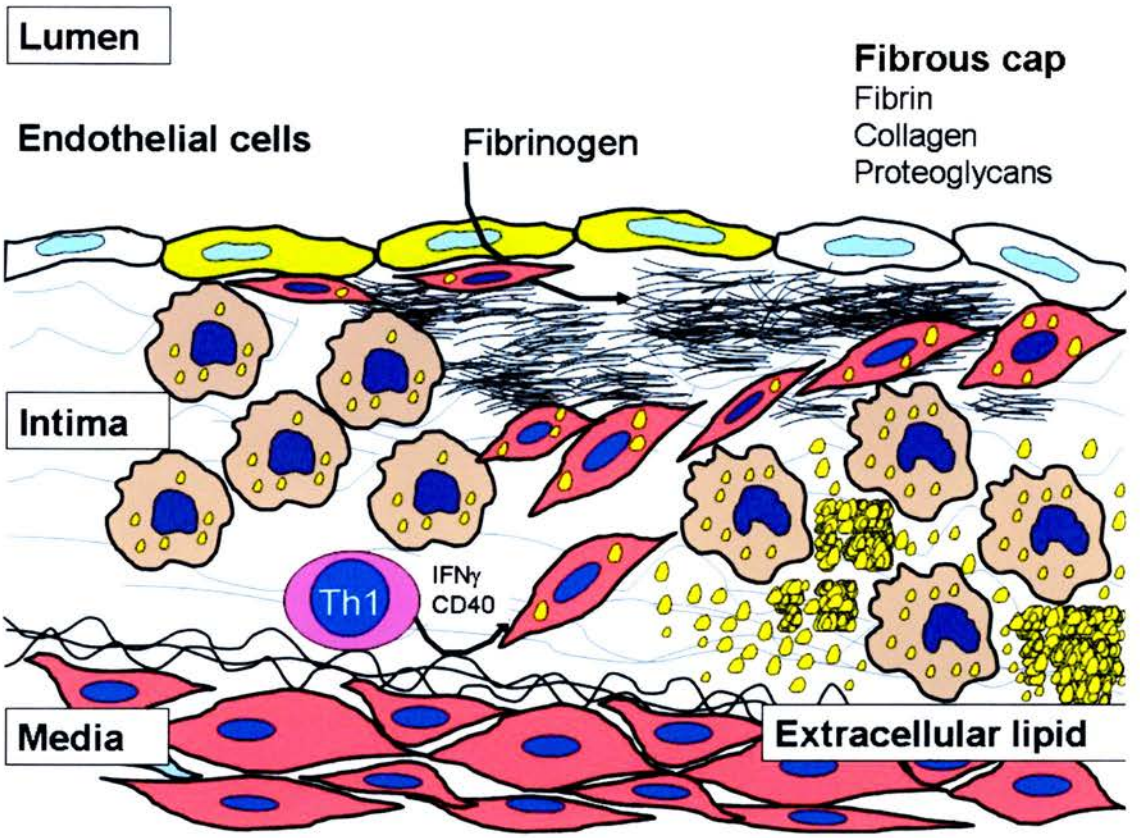


Figure 1-10 Inflammatory influences increasing plaque complexity

Free intimal lipid pools may reflect overwhelmed macrophage lipid-uptake capacity. Smooth muscle cells may also ingest lipid, adopting a foam cell phenotype.

An increasingly organised fibrous cap is strengthened by fibrin derived from circulating fibrinogen, and further VSMC recruitment. The proteoglycans biglycan and decorin displace normal arterial collagen I and III fibrils.

Collagen and proteoglycans increase locally, increasing lesion bulk.
These changes broadly correspond to the Type V fibrotic plaques in the Stary classification, although lipid content in these lesions may be variable.

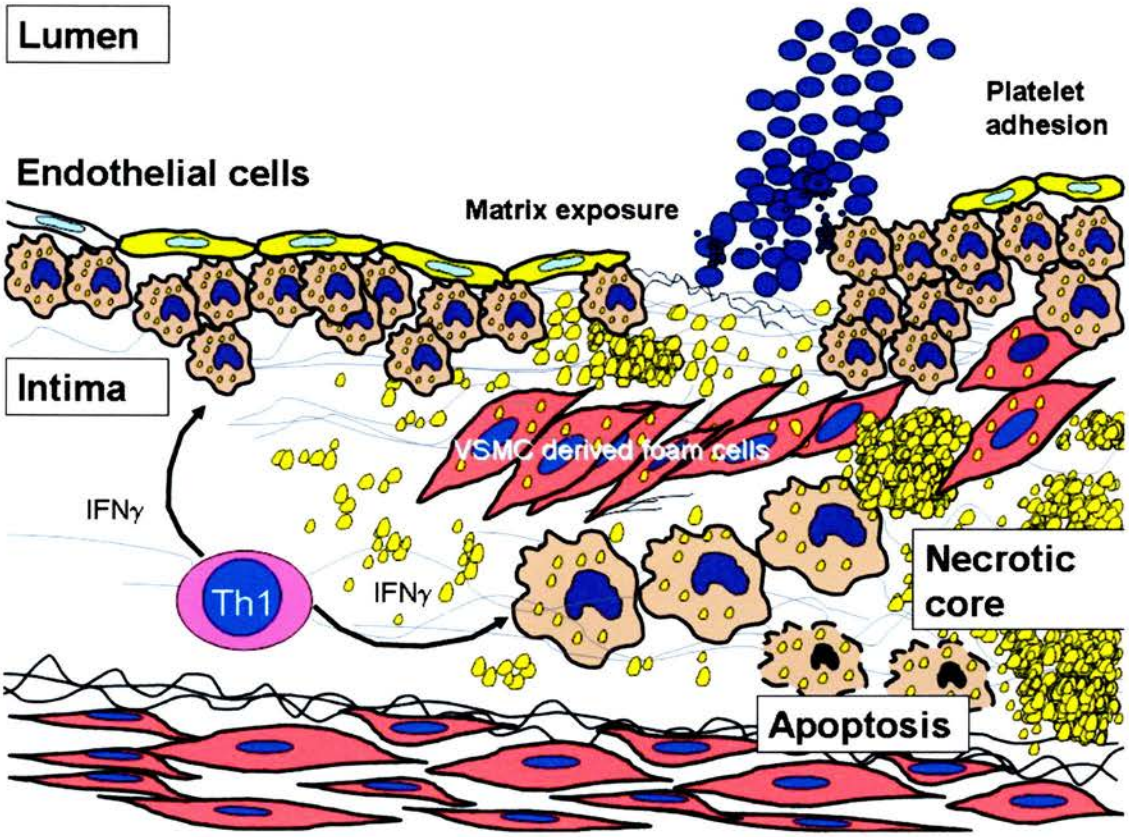


Figure 1-11 Inflammatory influences upon atherothrombosis

Accumulation of intracellular lipid promotes apoptosis of lipid-laden macrophages, contributing to free lipid within the necrotic core of the plaque.

VSMCs also ingest lipid, adopting a foam cell phenotype.

Elevated macrophage infiltration of plaque shoulder regions is accompanied by high levels of MMP activity, predisposing to proteolytic degradation, and potential plaque erosion, fissuring or rupture.

Exposure of both vascular matrix collagen, and the plaque necrotic core, to intra-luminal blood enhances platelet deposition and thrombus formation. Contact with free lipid components including lysophosphatidic acid is a further pro-thrombotic stimulus.

These events correspond to AHA type VI lesions that may be clinically symptomatic.

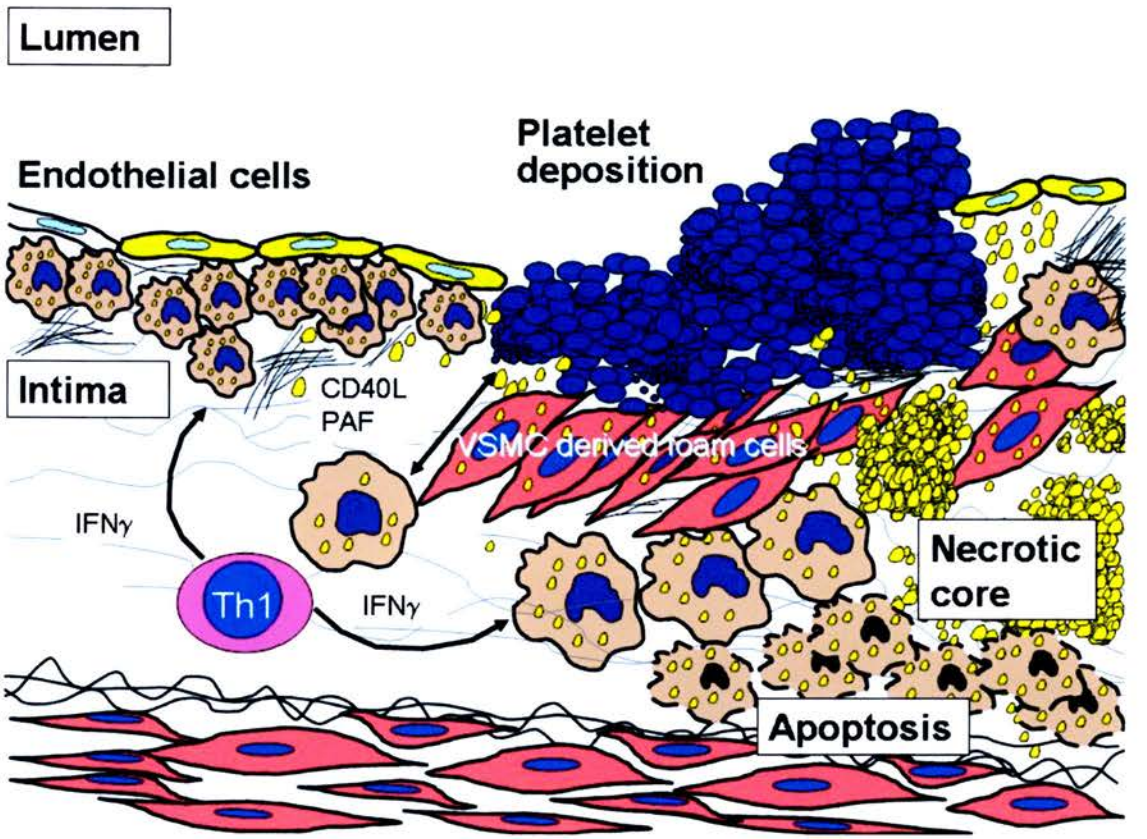


Figure 1-12 Inflammatory sequelae of continued atherothrombotic events

Platelet activation compounds local inflammatory activity via interactions between the platelet surface adhesion molecule P-selectin and macrophage surface P-selectin glycoprotein ligand-1 (PSGL-1).

Signalling between platelet GPIbα and the leukocyte Mac-1 integrin exacerbates leukocyte mediated vascular damage, and platelet-neutrophil interactions may be specifically important in this regard.

Platelet-derived growth factor (PDGF) may enhance VSMC proliferation and lesion bulk during lesion resolution.

This suggests that platelet-derived signals propagate inflammatory vascular damage resulting in neo-intimal hyperplasia during inflammatory repair. Thrombotic incorporation into resolving plaque inflammation may occur, further contributing to lesion volume.

Unregulated inflammation and plaque rupture correspond to Stage VI of the Stary/AHA classification, and will be clinically symptomatic dependent on reductions in vascular flow.

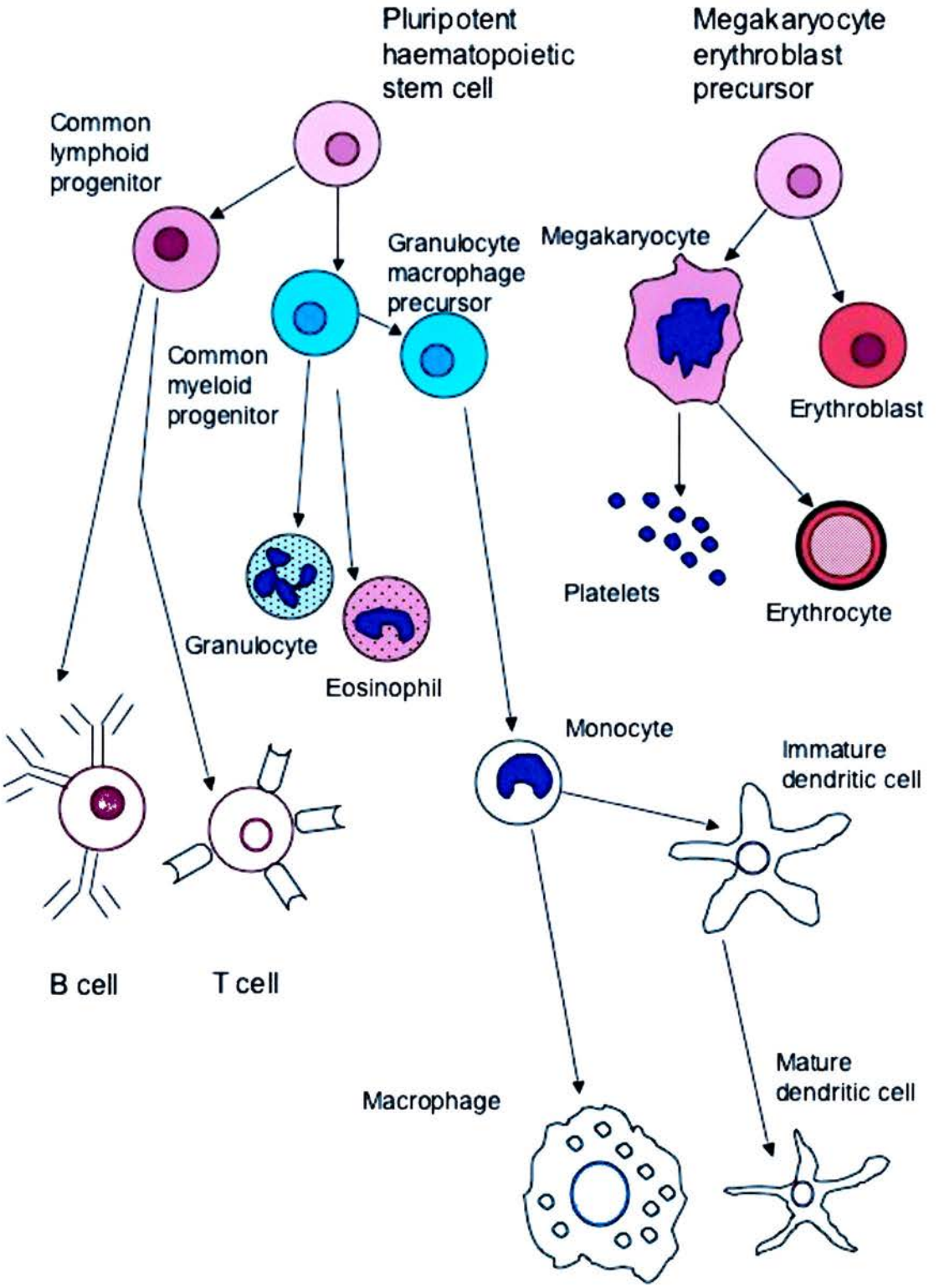


Figure 1-13 Myeloid cell precursor differentiation schematic

Adapted from Immunobiology, 5th edition Janeway et al, 1999.

Chapter 2 MATERIALS AND METHODS

2.1 General materials

Affinity Research Products, Exeter, UK:

Δ12PGJ$_2$ & 15-deoxy12PGJ$_2$ (both dissolved in DMSO at 100mM, stored at -20°C.)

z-VAD-fmk methyl ester.

Ambion Europe Huntingdon UK:

DNase I, 10x DNase buffer, and DNase inactivation reagent

Diethylpyrocarbonate (DEPC) treated water

Nuclease-free water

Chromogenix AB, Sweden:

Limulus amoebocyte lysate-based COATEST® endotoxin analysis system.

Amersham Pharmacia Biotech, Bucks, UK:

Ficoll-Hypaque

Horse-radish peroxidase-conjugated donkey anti-rabbit immunoglobulin antibody.

Hybond C nitrocellulose membrane.

^{33}Phosphorus labelled dATP.

Baxter Healthcare Ltd., Glasgow, UK:

DiffQuik stain, solution I (Eosin G in phosphate buffer, pH6.0)

Solution II, (Thiazine blue in phosphate buffer; pH6.0).

Sterile normal saline solution (0.9%)

Becton Dickinson, UK.

Tissue culture plasticware/polypropylene tubes.

Bender MedSystems (Vienna Austria):

Fluorescein Isothiocyanate (FITC)-labelled recombinant human Annexin V

Calbiochem USA:

Mowoil

Cell Signalling Technology MA USA.

Polyclonal rabbit anti-IκBα antibody

Clontech Laboratories, BD Biosciences, Palo Alto, CA, USA:

Atlas Nucleospin extraction kit.

Atlas pure total RNA Labelling system.

Atlas Cardiovascular nylon membrane gene array.

Gibco Life Technologies Paisley UK:

30% (w/v) acrylamide/bis solution.

Culture supplements: penicillin (50 U/ml)/streptomycin (50 U/ml); L-glutamine (200mM)

10% SDS

Hanks Balanced Salt Solution (HBSS)

Iscove's Dulbecco's modified Eagles medium without supplements with L-glutamine (Iscove's DMEM)

SuperScript II RNase H Reverse Transcriptase.

Taq DNA polymerase, recombinant.

Leo Laboratories, UK

Unfractionated heparin sodium, 1000U/ml

Martindale Pharmaceuticals Ltd Romford UK:

Calcium chloride

Millipore MA USA:

Immobilon PVDF membrane

Miltenyi Biotech Bergisch Gladbach, Germany:

CD61 microbeads

LS+ ferromagnetic cell separation columns.

MACS monocyte isolation system

Molecular Dynamics, Sunnyvale CA, USA:

Image Quant phosphorimager analysis software.

Perimmune/Intracel Corporation, Maryland USA:

Oxidised, native and acetylated low density lipoprotein, (unlabelled and 3,3'-dioctadecylindocarbocyanin-iodide (Di-I) labelled).

Pharmacia Fine Chemicals, Uppsala, Sweden:

Percoll.

Phoenix Pharmaceuticals Gloucestershire UK:

Sodium citrate solution

Promega Corporation WI USA:

1kb DNA ladder.

dNTP mix (dATP, dCTP, dGTP, dTTP).

0.1M DTT.

$MgCl_2$

Oligo dT 15 Primer

pSP72 vector.

SmaI, XhoI, XbaI restriction enzymes.

Taq DNA polymerase in Storage Buffer A.

T4 DNA ligase;

R&D systems Europe Oxon UK:

TNF alpha (stock solution 10μg/ml).

Sigma chemicals UK, further reagents:

Acrylamide

0.1% Bovine Serum Albumin.

Bromophenol blue.

100% Chloroform

Dextran-500, (dissolved in sterile 0.9% saline, 6% w/v, stored at 4°C until use.)

Dimethyl sulphoxide (DMSO)

Dithiothreitol (in H_2O at 100μg/ml stored at 4°C)

Dulbecco's phosphate buffered saline (PBS) sterile, endotoxin-free, pH7.4 with and without 1.2mM Ca^{2+} and 0.8mM Mg^{2+}.

Ethanol, 100%

Ethidium bromide

Ethylenediaminetetraacetic acid (EDTA)

Harris haematoxylin.

Hoechst 33248, and 33342.

LPS, dissolved in PBS at 1 mg/ml, sonicated (ultrawave sonic bath, Belmont Instruments Glasgow).

Oil Red O powder.

Paraformaldehyde

Propan-2-ol (isopropyl alcohol)

Propan-1,2-diol (propylene glycol).

Propidium iodide.

Prostaglandin J_2 (in ethanol at 2.99mM stored at -20°C).

Protease inhibitors:

Aprotinin (in H_2O at 100μg/ml stored at -20°C)

Leupeptin and pepstatin A (in methanol at 100μg/ml stored at -20°C).

4-(2-amonoethyl)-benzenesulphonyl fluoride (in H_2O at 50 mM at 4 deg C)

Sodium orthovanadate, (in H_2O at 100mM at -20°C).

Benzamadine (in H_2O at 50mM stored at -20°C)

Levamisole (in H2O at 200mM at -20 deg C).

0.1% sodium azide

TRIZMA base

Trypan blue.

2.2 Monoclonal antibodies

Protein blotting

PPARγ, clone 4.17.2 (gift), GlaxoWellcome, Stevenage UK.

PPARγ, clone E8 Santa Cruz Biotechnology Inc., (USA),

Ser-32 phospho I-κB New England Biotechnologies, USA.

Flow cytometry

Epitope	Clone	Isotype	Manufacturer/source
IgG_1	W3/25		Serotec, Oxon, UK
IgG_{2a}	MRC OX-34		Serotec, Oxon, UK
CD11a	Wac70	IgG_{2a}	Dr J Ross, University of Edinburgh
CD11b	Clone 44	IgG_1	ICRF, London UK
CD11c	3.9	IgG_1	ICRF, London UK
CD14	UCHM1 MEM18	IgG_1 IgG_1	ICRF, London,UK Serotec, Oxon, UK
CD16	3G8	IgG_1	JC Unkeless, Rockefeller University, New York
CD29	12G10	IgG_1	Serotec, Oxon, UK
CD36	smϕ	IgG_1	ICRF, London UK
CD36	IVC-7	IgG_1	Pelicluster; Amsterdam, NL
CD47	B6H12	IgG_1	Abcam, UK
CD49d	44H6	IgG_1	Serotec, Oxon, UK
CD49e	JBS5	IgG_1	Serotec, Oxon, UK
CD54	Clone 15.2	IgG_1	ICRF, London UK
CD61	PM6/13	IgG_1	Serotec, Oxon, UK
CD64	10.1	IgG_1	Serotec, Oxon, UK
CD86	BU63	IgG_1	Caltag, Burlingame CA, USA
Class II	WR18	IgG_{2a}	Serotec, Oxon, UK
Goat anti-mouse immunoglobulin fluorescein isothiocyanate (FITC)		$(F(ab')_2$ fragment)	Dako, UK.

2.3 Methods

2.3.1 Monocyte isolation

2.3.1.1 Discontinuous density centrifugation

Human peripheral blood leukocytes were isolated using discontinuous density centrifugation (Dransfield et al. 1994; Szefler et al. 1987). With local ethical approval, in accordance with the Declaration of Helsinki, whole blood was obtained by venepuncture using 19 gauge needles (Sarstedt UK), from healthy volunteers with no history of inflammatory disease, and on no current drug therapies. 36 ml whole blood was collected into 50ml Falcon polypropylene tubes containing 4mls of 3.8% sodium citrate pre-warmed to room temperature to anti-coagulate samples. Samples were gently inverted to mix with minimal cell activation, and then centrifuged at 350*g* at room temperature for 20 minutes. Platelet-rich plasma was separated from the leukocyte and erythrocyte pellet, which was re-suspended in sterile normal saline at 37°C with the addition of 5mls of 6% Dextran (T500) to promote erythrocyte sedimentation. Samples were allowed to sediment for 30 minutes at room temperature, before removal of the now well-defined upper leukocyte layer. This was further centrifuged at 350*g* at room temperature, and the supernatant discarded leaving a leukocyte pellet, which was resuspended in 2.5mls of 55% isotonic Percoll (9:1 vol/vol Percoll:10xPBS). Discontinuous gradients were formed by overlaying 2.5ml of a 68% Percoll mixture over 2.5ml of a 79% Percoll mixture, before careful overlaying of the 55% Percoll/leukocyte mixture on top. Gradients were centrifuged (720*g*, 20 minutes) resulting in distinct leukocyte bands forming: an upper mononuclear cell layer and a lower polymorphonuclear granulocyte layer. Mononuclear cells were carefully harvested by aspiration, using a sterile disposable Pasteur pipette. Mononuclear cells were resuspended in PBS and cell yield was assessed with a haemocytometer. Volunteers were screened to ensure that they did not have elevated eosinophil levels, by flow cytometry and light microscopic assessment of cytocentrifuge preparations stained with haematoxylin/eosin according to previous protocols (Ward et al. 1999).

2.3.1.2 Ficoll-Paque centrifugation

An alternative method was used to produce a granulocyte-free mononuclear cell preparation. Mononuclear cells were isolated using centrifugation through Ficoll-Paque®. Freshly drawn human venous blood was anti-coagulated with heparin at 10U/ml. Cells were diluted with 2 volumes of cation deplete phosphate buffered saline. 35 mL of diluted cell suspension were layered over 15mL Ficoll-Paque® in a 50mL conical tube and centrifuged at 400*g* for 30

minutes at 20°C. The upper layer was aspirated, leaving the mononuclear cell layer undisturbed at the interphase. The interphase was then aspirated and deposited in a fresh 50mL conical tube. Cells were washed with cation-depleted PBS and centrifuged at 300*g* for 6 minutes at 20 °C. This step was repeated to enhance removal of platelets.

For both discontinuous gradients and Ficoll-paque separation, cell purity was assessed by flow cytometric scatter properties. 20µl samples of isolated cell suspensions were used to quantify individual leukocyte populations, before proceeding to further purification.

2.3.1.3 Further monocyte purification: immunomagnetic separation

Further monocyte purification was performed either by using *in vitro* adhesion to tissue culture plastic, and/or by immunomagnetic separation.

For adhesion mediated purification without prior immunomagnetic bead separation, cells suspended in Iscove's DMEM at a concentration of $4x10^6$ per ml were allowed to adhere to tissue culture plastic (Falcon or BioWhittaker, UK), for one hour at 37°C/5%CO_2. Non-adherent cells were removed by circumferential washing of cell culture wells three times with cation replete PBS, using sterile plastic pastettes.

Immunomagnetic purification was performed using a magnetic bead separation technique (MACS, Miltenyi Biotech GmBH Germany). Mononuclear leukocytes, isolated either by Ficoll-Hypaque or Percoll density gradient centrifugation, were incubated with 5% autologous plasma, prepared from platelet rich plasma by centrifugation at 1000*g* for 5 minutes on ice, to block potential Fc receptor binding. Leukocytes were incubated with monoclonal antibodies to CD3, CD7, CD16, CD19, CD56 and glycophorin A, in a pre-prepared cocktail (Miltenyi Biotech GmBH Germany) for 15mins. Cells were washed in cation-deplete PBS/5% plasma before repeated plasma incubation for 10mins on ice. Target leukocytes were incubated with hapten conjugated beads. Leukocytes were filtered through a 30 micron pore-diameter sterile mesh and pipetted onto a ferromagnetic cell separation column (LS+ column, Miltenyi Biotech GmBH), held in a magnetic field. Eluate was thus composed purely of monocytes, with lymphocytes, megakaryocytes and natural killer cells being left attached to the elution column. Purity post-elution consistently approached 90%. Cell numbers retrieved were consistent with the estimated monocyte sub-population percentage ascertained by flow cytometric scatter properties prior to purification.

2.3.2 Primary human monocyte culture

2.3.2.1 Adhesion based experiments

Monocytes were plated onto tissue culture plastic (BioWhittaker, UK) at $1x10^6$ cells/ml in Iscove's DMEM for 1 hour, washed with PBS plus cations, and cultured in Iscove's DMEM plus 10% autologous serum at 37°C/5%CO_2. Monocytes were cultured for relevant time periods from 24 hours to 14 days. Prolonged incubation necessitated the replenishment of culture medium and serum and treatment supplements every 48 hours.

2.3.2.2 Suspension culture experiments

For suspension culture, monocytes were re-suspended at $1x10^6$ cells/ml, in Iscove's DMEM plus 10% autologous serum. Cells were then aliquoted into 5, 7 or 15 ml Teflon pots (Tuf Tainer, Pierce Chemical USA/Perbio UK), and cultured at 37°C/5%CO_2. Cell number was verified at sampling time-points to assess cell loss, and trypan blue exclusion was used to assess cell membrane integrity, as a marker of a lack of necrosis during culture.

2.3.2.3 Apoptosis response experiments

Monocytes were cultured adherent to tissue culture plastic in serum free conditions using Iscove's DMEM, or X-Vivo 10 medium (BioWhittaker, UK). Cells were incubated at 37°C/5%CO_2.

2.3.3 Culture of THP-1 monocytic cell lines

THP-1 human monocytic leukaemia cells were sourced from the European Collection of Cell Cultures, Salisbury Wiltshire UK (cell line no. 88081201). Cells were rapidly thawed and resuspended in 4ml of RPMI medium supplemented with 20% foetal bovine serum and 2mM glutamine in a 20ml conical tube. The cell suspension was centrifuged at 150g for 5 minutes. THP-1 cells were resuspended at a density of $5x10^5$ cells/ml in fresh medium in 200ml tissue culture flasks, incubated at 37°C/5%CO_2, until the cells reached the exponential phase of growth. Once cultures were established the serum concentration was reduced to 10%. Cell numbers were monitored on a daily basis, and kept at a constant concentration of $5x10^5$/ml to maintain exponential growth.

2.4 Electrophoresis

2.4.1 Buffers for protein electrophoresis

Materials were purchased from Sigma (Poole, Dorset) unless specified

Triton X-100 Lysis buffer

10mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, pH to 7.4 with conc HCl, one tablet protease inhibitor cocktail per 20ml buffer.

NP-40 lysis buffer

1% NP-40 non-ionic detergent; 0.5% sodium deoxycholate; 0.05M Tris pH 8.0; sodium chloride 0.15M; protease inhibitor cocktail

Blocking buffer

TBS or PBS with Tween-20 0.1%; 5% non-fat milk-protein (Marvel) freshly prepared.

Stacking buffer(x4)

0.5M Tris, pH adjusted to 6.8 with conc. HCl.

Separating gel buffer(x4)

1.5M Tris, pH adjusted to 8.8 with conc. HCl.

SDS-Tris glycine electrophoresis buffer (x10)

0.25M Tris-HCl, 1.92M glycine, 1% SDS stored at room temperature.

Acrylamide stock:

30% Acrylamide/0.8% Bis-acrylamide in 250ml dH_2O, filtered through Whatman No1 paper, stored in darkness at 4°C.

9 % gels

2.5ml 4x Separating buffer; 3ml acrylamide stock; 50µl SDS; 20µl TEMED; 40 µl 10% ammonium persulphate; 4.4ml dH_2O.

12% gels

2.5ml 4x Separating buffer; 4ml acrylamide stock; 50µl SDS; 20µl TEMED; 40µl 10% ammonium persulphate; 3.4ml dH2O.

Equilibration/Sample buffer

2.5ml 4x stacking gel buffer, 2ml 20% SDS, 5.5ml 50% glycerol, 20µl Bromophenol blue (1% w/v in ethanol).

Methanol Based Transfer Buffer

0.192M glycine, 0.025M Tris-HCl, 20% Methanol by volume. Freshly prepared

2.4.2 Immunoblotting

Acrylamide gels were cast using a BioRad casting stand and cleaned glass plates fitting a "Mini-gel" system, (BioRad UK). Running gel layers were cast at room temperature, and after pouring were layered with 10µl of ethanol to allow clean setting. The alcohol was removed after setting was complete. A 4.5% stacking gel solution was poured, and a pre-

formed 1mm gel comb inserted between glass panels. Gels were allowed to finally set at room temperature, and were used immediately after casting. Gels were run in tandem using a proprietary BioRad gel tank, and running buffer. Standard protein ladders were used to gauge protein size (Gibco Life Sciences, Paisley UK). 20µl aliquots of sample in sample buffer were pipetted into separate wells using a Hutchinson needle. Slow electrophoresis was used through stacking layers, with more rapid electrophoresis through running gel layers. Standardised transfers were performed with Hybond nitrocellulose membrane or PVDF membranes according to proteins being examined, with the latter being pre-wetted in methanol. Transferred blots were processed using ECL or ECL plus reagents (Amersham, UK), before wrapping in Saran-Wrap and exposing to photographic film (Kodak, UK).

2.4.3 LDL electrophoresis

LDL samples were analysed by electrophoresis using a semi-automatic Hydrasys system designed for the use of Hydragel agarose gels (Sebia, Issy-les-Moulineaux, France), under the supervision of Dr G Beckett, Clinical Chemistry, New Royal Infirmary Edinburgh (Dr Beckett prepared the hydragel agarose gel with 8g/L agarose in Tris barbital buffer, pH 8.5±0.1).

10µL aliquots of LDL fractions were applied to the gel and electrophoresis carried out at a constant 10W for 7 min at 20°C. The plate was dried for 10min at 65°C. The gel was developed using a proprietary Sebia Amidoblack staining solution (4g/L), destained with Sebia's destaining solution and dried. The gel was then photographed.

2.5 Flow cytometric analysis

Isolated monocytes were prepared by re-suspending at $2x10^6$/ml in flow buffer (PBS with cations, 0.1% BSA 0.1% sodium azide) and then washing in flow buffer in Immunotech flexible plates, followed by centrifugation at 200*g* for 3 minutes at 4°C. Non-specific Fc receptor binding was avoided by blocking samples with 5% heat-inactivate human AB serum or 5% normal rabbit serum for 5 minutes on ice. Cells were labelled with un-conjugated monoclonal antibodies, used at saturating concentrations as determined by flow cytometry, for 30 minutes on ice. Cells were then washed twice in flow buffer, before being resuspended and labelled with fluorochrome-conjugated secondary antibodies. Two further wash cycles in flow buffer were performed before cell pellets were resuspended in 100µl of flow buffer. Alternatively, for Annexin V binding assays, cells were resuspended in cation-replete buffer (PBS with 5mM $CaCl_2$) and incubated with 50µL of a 1:200 dilution of FITC-annexin V at 4°C for 10mins (Bender Med Systems). Cells were analysed using either a

Becton Dickinson FACSCalibur flow cytometer running CellQuest software (BectonDickinson UK) or a Coulter XL flow cytometer running EXPO 32 software (Beckman Coulter UK). Baseline fluorescence was measured on unlabelled cells and on isotype controls. Fluorescence was quantified as follows. Net mean fluorescence for each surface marker was calculated by subtracting the mean fluorescent intensity (MFI) for the appropriate isotype control (ΔMFI). The net difference of this figure for each treatment was then compared to control, with the net change being noted as a positive or negative value (ΔΔMFI).

2.6 Statistical analysis

The candidate attended the University of Edinburgh Department of Public Health medical and biostatistics course. All statistical analyses presented were performed following consultation with Dr Sarah Wilde, Senior Lecturer in Public Health, University of Edinburgh, and Dr Ernst Wit, Reader in Statistics, University of Glasgow.

Statistical analysis on fluorescence data for two variables was performed using the student's t test. Data were assessed for differences between standard deviations to ascertain if pairing was appropriate. If not, Mann-Whitney U tests were perfomed. For calculations of relative fluorescence, Wilcoxon signed rank tests are given to calculate deviation from a theoretical median of zero, and differences in fluorescence between individual treatments are calculated separately. For multiple treatment experiments, one-way analysis of variance tests (Tukey or Bonferroni methods) appropriate to sample size were used. If two variables were being assessed, e.g. in apoptosis data, then 2-way ANOVA was used as appropriate. Statistical significance is given as $p<0.05$.

2.7 Measurement of intracellular calcium

Fura-2 loaded monocytes at $1x10^6$/ml were equilibrated in a Perkins Elmer spectrophotometer at 37°C for 10 minutes, to achieve a stable baseline. The spectrophotometer was set to excitation wavelengths of 340 and 380nm with an emission wavelength of 580nm. A baseline recording was performed for 1 minute before the addition of cyclopentenone prostaglandin ligands, fMLP and digitonin. Calcium flux was recorded for 5 minutes and data analysed on FL Win software (Perkins Elmer USA).

2.8 Cell labelling and microscopy

2.8.1 DiffQuick labelling

Leukocytes were washed in PBS without cations, and 50μl droplets placed in cytocentrifuge chambers containing glass microscopy slides, with the addition of 5μl of serum to promote cell stability. After centrifugation at 300rpm for 3mins (Cyto-tek, Miles Scientific, USA), slides were immersed in methanol, and then sequentially in the two Diff-QuikTM stainsets for 1 minute each. Slides were washed in double distilled water and air-dried before mounting coverslips fixed in distrene plasticiser xylene (DPX) medium.

2.8.2 Oil Red O labelling

Monocytes were cultured at $1x10^6$/ml on glass coverslips in the base of 12 well tissue culture plate (BioWhittaker, USA), or in 8 well glass slide-chambers (Nunc Biosciences, USA). Iscove's preparation of Dulbecco's modified Eagle's medium was used with the addition of L-glutamine, penicillin and streptomycin, as for phenotyping experiments. Medium was supplemented with 10% autologous serum, with or without the addition of n-LDL or Ox-LDL at concentrations of 10, 50 and 100μg/ml. Adherent monocytes were washed with PBS with cations, and fixed with formaldehyde, before being washed briefly with propan-1,2-diol (propylene glycol). Powdered Oil Red O was dissolved in 65% propan-1,2-diol by heating at 95°C for 1 hour and passed through a 45 micron filter, and cooled to room temperature. Oil Red O solution was applied to coverslips or slide chambers for 20 minutes at room temperature, whilst slowly agitating coverslips or slide chambers on a flat-bed shaker. Slides were washed with 65% propan-1,2-diol at room temperature before counterstaining with Harris haematoxylin for 1 minute. Final conversion of haematoxylin was performed using Scott's tap water solution (magnesium sulphate 29mM; sodium bicarbonate 42mM). Slides were rinsed in double distilled water processed through a MilliQ filter, before mounting coverslips. Slides were imaged immediately.

2.8.3 Fluorescent lipoprotein labelling

Monocytes were cultured at $1x10^6$/ml in identical conditions to cells used for Oil Red O labelling, with the exception of the type of LDL supplementation used. Di-I labelled n-LDL and ox-LDL was used to supplement culture media, and cells grown in adherent culture for up to 8 days. Cells were fixed in methanol-free 3%w/v para-formaldehyde/phosphate-buffered saline. Nuclei were counterstained with Hoechst 33248, and coverslips or slides were washed and fixed in Mowoil for fluorescent imaging.

2.9 Microscopy

2.9.1 Light microscopy

Light microscopy was used to image monocytes labelled with DiffQuik or Oil Red O as described in sections 2.8.1 and 2.8.2. Images were captured using a Zeiss Axiovert microscope, using x10, x20, and x32 lenses. A CCD digital camera enabled multiple images to be taken with different fluorescent labels. Image processing was carried out using OpenLab 3.15 or 4.01 software, utilising automated time exposure and contrast-balancing.

2.9.2 Fluorescent microscopy: assessment of apoptosis and LDL uptake

A Zeiss Axiovert microscope connected to a fluorescent light source was used with x10, x20, x32 and x100 lenses, the latter in oil immersion conditions.

Hoechst nuclear labelling was imaged using a DAPI filter for assessment of apoptosis. Cell samples were blinded to the operator, and a key used to decode cell counts. 500 cells per field were counted, with cell nuclear morphology used to define apoptosis. Apoptotic bodies/ "ghosts" were also counted, defined by visible cytoplasm, but with a total absence of nuclear fluorescence. Percentage cell death was calculated per treatment.

For assessment of Di-I LDL uptake, monocytes were visualised using phase contrast and then with a rhodamine filter set, enabling imaging of fluorescence between 550 and 565nm, the excitation and emission wavelengths of Di-I. Counter-labelling with Hoechst 33248 enabled nuclear imaging for these preparations also.

Post-hoc image processing was carried out using OpenLab 3.15 or 4.01 software.

2.9.3 Confocal microscopy

Monocytes were grown in adherent culture supplemented with Di-I labelled n-LDL or ox-LDL, as for fluorescent microscopy. Monocytes were fixed in methanol-free 3%w/v para-formaldehyde/phosphate-buffered saline. Nuclei were counterlabelled with ToPro3, (Molecular Probes USA). Cells were observed using a x40 oil immersion objective lens with a numerical aperture of 1.2 on a Zeiss confocal laser scanning microscope system (Carl Zeiss AG, Jena, GmBH). Single optical sections of the images captured with Zeiss LSM software were digitally processed using Adobe Photoshop CS8.

2.9.4 Electron microscopy

Monocytes isolated by density gradient centrifugation followed by immunomagnetic separation were cultured in suspension in 10ml Teflon pots. Culture media were supplemented with 10% autologous serum, with or without LDL. Cells were matured for 7

days, before being washed in warm PBS with cations, and resuspended at $1x10^6$/ml. Cells were fixed in 2% glutaraldehyde in PBS/0.1M sodium cacodylate for 15 minutes, and centrifuged at 5,000 rpm for 5 mins to form a cohesive pellet. Cell pellets were set in 1% high strength agar/distilled water to form plugs for section preparation. With the assistance and supervision of Mr S Mitchell, Royal Dick Veterinary School, University of Edinburgh, cell plugs were cut into 1mm cubes and placed in 0.1M sodium cacodylate buffer. Sections were made using a diamond ultra-microtome set to cut at 100nm using heat advance. Sections were picked up onto 300-mesh (300 squares), thin-bar, copper grids and examined using a Philips CM12 transmission electron microscope. Photomicrographs were produced with a magnification of x10,000.

2.10 Microarray methods

2.10.1 Monocyte preparation for mini array analysis

Human peripheral blood mononuclear cells were isolated as described above, from citrate-anti-coagulated whole blood separated by density gradient centrifugation. Purity was maximised by magnetic bead separation, monocytes attached to tissue culture plastic at 4x106 cells/ml in Iscove's DMEM for 1 hour and washed with PBS plus cations. Monocytes were cultured in Iscove's DMEM plus 10% autologous serum supplemented with ox–LDL at 50μg/ml. Monocytes were incubated at 37°C/5%CO2 for 24 hours before carefully removing supernatants, rinsing briefly in cation replete PBS at 37°C, and lysing with Trizol.

2.10.2 Mini-array preparation

RNA was prepared by chloroform separation and centrifugation, followed by precipitation and washing with isopropanol and ethanol according to standard RNA preparation procedures (see section 2.13). Purity and sample mass was checked by a combination of absorbance spectrometry and agarose gel electrophoresis of samples run against standard molecular weight makers, Hind III digests, (Promega, UK), and low molecular mass DNA ladders (Life Sciences Paisley, UK). Samples were treated with DNase I to remove contaminating genomic DNA, which would reduce probe purity and sensitivity. For each microlitre of RNA isolated and resuspended in nuclease free distilled water, 0.1μl of 10x DNase I buffer was added in a 0.5ml Eppendorf container. 2 units of DNase I enzyme were added, and the reagents gently mixed, prior to incubation for 30 minutes at 37°C, before inactivation of the enzyme for 2 minutes at 37°C in $1/10^{th}$ of the reaction volume of an EDTA based inactivation buffer (Ambion, UK) to stop enzyme activity. Samples were subject to centrifugation at 16,110*g*, and the supernatant containing DNase I treated RNA

was aspirated. Sample purity was again checked by spectrophotometry and agarose gel electrophoresis.

2.10.3 Mini-array probe synthesis

Between 2 and 5μg of total RNA was used to synthesise ^{32}P radioactively labelled first-strand cDNA. A "master mix" was prepared as for reverse transcriptase polymerase chain reaction (RT-PCR) work (Section 2.10.8.1), using 2μl of 5x reaction buffer (Clontech USA) per sample. 1μl of 10x dNTP mix was added to this, plus 3.5μl of α-^{32}P dATP, at 3,000 Ci/mmol (10μCi/μl). 0.5μl of 100mM dithiothreitol per sample was then added before addition of 1μl of Mouse Moloney Leukaemia Virus reverse transcriptase (MMLV-RT). In a separate tube, 1-2μl of RNA sample was added to 1μl of a 10x solution of array gene-specific cDNA primer mix, and made up as necessary to a final volume of 3μl with nuclease free distilled water. The sample/primer mix was heated to 70°C for 2 minutes before cooling to 50°C for a further 2 minutes. 8μl of the Master Mix previously prepared was then added to each reaction, and this was allowed to incubate at 50°C for 25 minutes, before the reaction was stopped using 1μl of 10x termination mix (0.1M EDTA, pH 8.0, 1 mg/ml glycogen).

2.10.4 Mini-array probe isolation

Purification of synthesised probe was carried out initially using modified gel filtration columns. This process used sequential elutions through gel columns, which were prepared at room temperature by pre-wetting with distilled water. Samples were then applied to the flat surface of each gel column, and then rinsed with 40μl of distilled water, followed by a further 250μl of distilled water. These wash fractions were discarded. Columns were then consecutively placed over 6 separate 1.5ml Eppendorf tubes, and 100μl of distilled water was applied to the column surface. Each 100μl fraction was collected by gravity, before the next aliquot of water was applied. Samples were collected into each of the different Eppendorf tubes. 2μl aliquots of each sample were then added to 5ml scintillation-counter vials, and the volume made up to 5ml with scintillation fluid. Each vial was then assayed using a ^{32}P channel protocol using a Packard 1900 CA scintillation counter (Packard Biosciences, Pangbourne, Berks UK). Elution profiles were plotted using data from the counts, and estimates of activity of each fraction drawn from these figures. In a properly incorporated probe, an initial peak of activity in fraction 2 and 3 is seen. This is followed by a low activity eluate and then a high activity peak in fractions 5 and 6 indicating unincorporated nucleotide. Eluates prepared by this method did not show this profile, and for this reason, an alternative method of probe isolation was employed. Active probe was

bonded onto the surface of a micro-centrifuge tube containing a silica membrane in conjunction with a chaotropic salt, enabling nucleic acid binding (Clontech USA). After absorption of nucleic acids to the membrane, cellular debris was centrifuged at 16,110*g* through the column and discarded. Purified nucleic acid was eluted from the membrane in a proprietary elution buffer (Clontech USA), by centrifugation at 16,110*g* at room temperature for 1 minute, and collected as a single fraction.

Samples were assayed for activity by a scintillation counter, and relative activity to an internal poly A+ positive control was assessed. Counts within the recommended range were deemed acceptable. To minimise differences between individual probe activities, probes were diluted to the same activity per unit volume before adding to hybridisation buffer.

2.10.5 Mini-array hybridisation

Membranes were handled carefully to avoid tearing or distorting the arrayed surface. For pre-hybridisation of membranes, 0.5mg of sheared salmon testes DNA (SS-DNA) was heated to 99-100°C, and then immediately chilled on ice. Proprietary hybridisation buffer (Clontech USA) was pre-warmed in a water bath to 68°C. 5ml of hybridisation buffer was mixed with SS-DNA and held at 68°C until use. The nylon membrane arrays were pre-wetted with distilled water, and surplus water shaken off. Arrays were then rolled carefully and placed in Hybaid hybridisation bottles (Thermo Electron Inc., USA), and 5mls of pre-hybridisation buffer containing SS-DNA evenly poured over them. Pre-hybridisation was carried out for 30 minutes with bottles rotating at 7 rpm at 68°C. To prepare isolated probe for hybridisation, 200µl of probe was added to 22µl of 10x denaturation buffer (1mM NaOH, 10 mM EDTA), and incubated for 2 minutes at 68°C. 5µl of Cot-1 DNA was added to 225µl of neutralisation solution (1M NaH2PO4, pH 7.0), and this mixture added to the incubating denatured probe at 68°C. This mixture was incubated for a further 10 minutes. Isolated, denatured and neutralised probe was then added directly to the hybridisation buffer at the base of the hybridisation bottle taking care not to coat the membrane directly with probe. Once probe had been mixed with hybridisation buffer, an extra 20 ml of pre-warmed buffer was added to each bottle to ensure adequate coating of the membrane surface. Bottles were rotated at 6-10 rpm at 68°C in a hybridisation oven for 16 hours. Prior to exposure of array membranes to probe, blank membrane was exposed to radioactive probe under the same hybridisation conditions used for actual array analysis. This was performed using blank membrane fragments, and hybridising for 12 hours. Blank membranes were exposed in the same manner as actual arrays, using autoradiography or storm analysis. Blank membranes were used to indicate the possible presence of contaminating genomic DNA within the

samples and the level of non-specific background due to binding to the membrane material directly. Following hybridisation, membranes were drained of hybridisation buffer, which was discarded according to radiation disposal guidelines. 200ml of a pre-warmed wash solution-1 (2x SSC, 1% SDS) was added to the hybridisation bottles, and this was used to wash membranes rotated at 15 rpm at 68°C for 30 minutes. This step was repeated 3 more times, and then two further 30 minute washes performed with wash solution-2 (0.1 SSC, 0.5 % SDS). A final wash using 200ml of 2xSSC at 15 rpm at room temperature was undertaken, before the membrane being removed and excess buffer being shaken off. The membrane was kept damp, and transferred into plastic film-wrap.

2.10.6 Mini-array membrane exposure and image acquisition

Array membranes were wrapped in Clingfilm, and mounted on Whatman 3MM Chromatography paper. Membranes were then taped onto a pre-drawn grid within a prepared phosphor imager screen cassette, and a blank phosphor imager screen clamped into the cassette. Membranes were exposed to the phosphor imager screen for 24 hours at room temperature, or Kodak BioMax MS film for 48-72 hours at –80°C. Internal control blank membranes were also exposed to active probe to ensure no significant background signal was present. These membranes were hybridised and exposed at the same time to ensure consistency of hybridisation and exposure conditions. Following exposure, membranes were stripped ready for re-probing. 500ml of a 0.5 % SDS solution was heated to boiling point in a glass beaker, and the unwrapped array membrane placed in the solution for 10 minutes. The solution was then cooled at room temperature for 10 minutes, and the membrane washed in a solution of 2xSSC, and 1% SDS. The stripped damp membrane was wrapped in fresh Clingfilm, and then re-exposed to a phosphor screen or film to assess stripping efficiency. After this the membrane was stored in Clingfilm at –20°C.

2.10.7 Mini-array software analysis

Two software analysis methods were used to read signals. ImageQuant software supplied by Molecular Dynamics was used to examine background radiation signals, by assaying local area background around each gene spot and and comparing signal to areas of blank membrane. Housekeeping gene expression was checked with this software package by assessing signal strength across all housekeeping genes, and averaging these signals.

Use of a Clontech Atlas Navigator software package was also made. This utilises pre-programmed grids to fit membrane array arrangements and has the option of eliminating poor quality signals from the analysis. Both methods were used, and compared.

Raw signal levels were taken and compared to housekeeping gene average signal levels, after background signals had been subtracted. Finally net signals were tabulated for each gene in a Microsoft Excel spreadsheet. The ratio of signals from ox-LDL treated samples was compared to that of control samples. Ratios were stratified into those showing over 2-fold changes, and those over 3-fold expression changes. Any significant changes were crosschecked with the actual appearance of membranes to ensure that apparently high ratio differences were based on good quality signals.

2.10.8 Confirmation of array transcriptional data

2.10.8.1 Reverse-transcriptase polymerase chain reaction

Initial confirmation of transcription changes was performed by RT-PCR, using separate donors and blood preparations. Monocytes were purified by density gradient centrifugation and adhesion to tissue culture plastic after magnetic bead separation using the negative selection protocol described (Section 2.3.1). Using Trizol prepared RNA from 24 hour old treated monocytes standard 1st strand synthesis was carried out using Superscript II reverse transcriptase (Gibco Life Sciences Paisley UK). 5μg of target RNA at 2 mg/ml was added to 1μl of oligo dT (Promega, UK), and 8.5 μl of nuclease free distilled water (Ambion Corp., USA). This was pre-prepared by heating to 70°C for 5 minutes and then chilling on ice, followed by a 15 second centrifugation at 16,110*g*. A Master Mix was made using 4 μl of 5x 1st strand reaction buffer, and 1 μl of dNTP mix at 10mM. 2 μl of Dithiothreitol was added to this followed by 1 μl of Superscript II (Gibco Life Sciences, Paisley UK). The entire contents of each volume of master mix were added to the prepared RNA sample, and incubated at 42°C for 60 minutes. Following incubation, the reaction was stopped by chilling on ice. 20% of the cDNA from the PCR reaction (4μl) was used as a template for a polymerase chain reaction, by adding 2.5μl of Mg-free PCR buffer, (Promega), 1μl each of forward and reverse gene specific primers and 16.5μl of nuclease-free distilled water. This was pre-heated to 94°C for 2 minutes and then cooled and held at 80°C. A polymerase cocktail was added to this mixture, containing 2.5μl Mg-free PCR buffer, 3 μl $MgCl_2$ at 25mM, 0.2 μl of Taq polymerase (Promega) and 18.8μl of nuclease free distilled water. PCR was carried out using a program allowing for primer dissociation at 96°C for 30 seconds, annealing at 62°C for 30 seconds and primer extension at 74°C for 30 seconds. This was carried out for 35 cycles. An assessment of product was made at 2 cycle increments across a range of cycle numbers from 18 to 30 to examine the linear range of transcripts. To stop the reaction, samples were held at 74°C for 5 minutes and then held at 20°C until analysis. 10μl

aliquots of each sample were run in 1.5 % w/v agarose gels made up with 1x Tris Borate EDTA (TBE) at pH 8.0, containing 0.1% by volume of Ethidium Bromide. Gels were cast and electrophoresed in iso-osmotic TBE to allow resolution of product bands. All samples were run alongside a 4μl of a 1kB molecular weight ladder (Life Sciences, Paisley UK). The internal control for cell housekeeping was G3 PDH (GAPDH).

Primer sequences used for LFA-1 α were as follows:
Primer 1 Forward: 5' CCT CGA GCT ACA ACC TGG AC 3'
Primer 1 Reverse: 5' GTT GGA ACC TCT CAG GGT GA 3'
Primer 2 Forward: 5' ACA GAC CCC ACA GAT GGA AG 3'
Primer 2 Reverse: 5' CAA GCT CAT CGA ACC ATC AA 3'

Primer sequences for CD47 were as follows:
Primer 1 Forward: 5' CTG CCT GTG ACG CGC GGC GTC 3'
Primer 2 Forward: 5' AGC GGC GCT GTT GCT GGG CTC GGC 3'
Common Reverse Primer: 5' GCA CCA TGG CCT TGA TGA TTC ACT 3'

Primer sequences for human GAPDH-S were as follows:
Forward: 5'-TGC CTC CTG CAC CAC CAA CTG C-3' Tm 65.8°C
Reverse: 5'-AAT GCC AGC CCC AGC GTC AAA G-3' Tm 64°C

2.10.8.2 Confirmation of array data at a protein level

Assessment of cell surface protein changes was performed by flow-cytometric analysis of cultured monocytes. Cells isolated by density gradient centrifugation and magnetic bead isolation were then cultured in suspension or adherent to plastic in Iscove's DMEM supplemented with 10% autologous serum. Lipid supplement of 50μg/ml Ox-LDL was added to experimental samples. Cells in suspension were cultured in Teflon pots, to allow for flow cytometry preparation. Cells in adherent cultures were removed from tissue culture wells by trypsin/EDTA treatment at 37°C for 5 minutes followed by washing in cold PBS without cations. Cells were pelleted, and cell surface expression determined by flow cytometric analysis (section 2.5).

2.11 Manipulation of PPARγ mutant gene

Wild type PPARγ and mutant (AF2) PPARγ were sourced in a pcDNA3 vector (Fig 2.1). Initial restriction digestion was performed using *XhoI* to release a PPARγ containing insert.

2.11.1 Restriction enzyme reactions:

Double digest of PPARγ and pSP72 vector (Fig 2.2)

XhoI	1µl
XbaI	1µl
BSA	2 µl
Nuclease free dH_2O	9 µl
Buffer D	2 µl
PPARγ or pSP72	Minimum of 1µg per reaction: volume of H_2O adjusted for DNA concentration

2.11.2 Gel purification of cut DNA fragments

Gel purification of ligation and restriction products was performed using common techniques described in detail in section 2.14. Gel fragments containing product of interest were resolubilised and used for further ligations, diagnostic restriction digests, or bacterial transformation. pIRES2-GFP vector was sourced from Clontech USA, (Fig 2.3).

2.11.3 Ligation of pSP72 cut fragments and PPARγ fragments

0.5µl T4 DNA ligase

1.0 µl DNA ligase buffer (Promega USA)

1:1 and 3:1 mass ratio of insert to vector, adjusted according to concentration

$$\left(\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \frac{\text{molar ratio of insert}}{\text{vector}} = \text{ng of insert}\right)$$

The reaction volume was made up to 10µl with Nuclease free water. The reaction was processed in a PCR cycler alternating between 15°C for 30 secs, and 24°C for 30 secs for 12 hours and then held at 4°C, allowing for latitude in ligation efficiency.

2.11.4 pSP72/PPARγ restriction digests with SmaI

Large volume digests of shuttle vector (pSP72/PPARγ insert) were performed with *SmaI*:

30 µl DNA

5 µl Buffer J/ Multi-core buffer (Promega, USA)

2 µl BSA

3 µl *SmaI*

10 µl nuclease free dH_2O

Allowed to digest overnight at 25°C.

2.12 Plasmid DNA isolation

2.12.1 Specific reagents for plasmid DNA preparation

Bacterial growth medium: Luria-Bertani lysogeny broth (LB Broth # BP1426, Fisher UK)

10 g tryptone

5 g yeast extract

10 grams of NaCl

Dissolved in 1 L of distilled water, autoclaved for 30 minutes.

2M Glucose filter sterilised and stored at 4°C.

RNAse A stock solution

50 mg Bovine Pancreas RNAse (Sigma R-6513)

Made up to 10 mg/ml in 10mM Tris (7.5) 15mM NaCl, stored at –20°C.

Used at 50 µg/ml

Glucose buffer (Buffer I)

1.25ml 2M glucose	50mM
1.25ml 1M Tris (pH 8.0)	25mM
1ml 0.5M EDTA	10mM
250 ml RNAse	50µg/ml

made up to 50 ml dH_2O, stored at 4°C.

Lysis buffer (Buffer II)

2ml 5N NaOH	200mM
5ml 10% SDS	1%

made up to 50 ml in dH_2O freshly as required

High salt buffer (Buffer III)

150ml 5M potassium acetate	3M
28.75ml glacial acetic acid	5M

made up to 250ml in dH_2O

LiCl solution

53g LiCl (MW 42.294)	5M
2.6g MOPS (MW 209.26)	50mM

made up to 250ml in dH_2O, pH adjusted to 8.0

Tris buffered phenol/chloroform

Pre-buffered phenol (Sigma P4557) added to equal volumes chloroform with $1/25^{th}$ volume Iso-amyl alcohol, stored in dark glass at 4°C.

TE Buffer

500 µl Tris (pH 8.0)	1M
100 µl EDTA (pH 8.0)	0.5M
made up to 50 ml in dH_2O	

Sodium Acetate solution

150 ml sodium acetate	5M
28.75 ml glacial acetic acid	5M
made up to 250 ml in dH_2O	

2.12.2 Bacterial transformation, plasmid preparation

50µl of transformation competent TOP10 *Escherichia coli* (Invitrogen) were thawed and chemically transformed using 5µg of relevant plasmid DNA added on ice and left for 30 minutes. Cells were thermally shocked at 42°C for 30 seconds before replacing on ice. Bacteria were then transferred to LB broth with ampicillin for initial culture. Preparation of DNA was done using either small-scale mini-preps or large volume bacterial preparations, using locally derived protocols (after Maniatis) or commercial plasmid preparation systems (Qiagen Midi-preps, Qiagen USA). In summary, fresh colonies grown on agar plates with appropriate antibiotic supplements were picked in an aseptic manner and mini-preps grown in 2ml volumes of LB broth. Bacterial cultures were grown overnight at 37°C with 200-250 rpm agitation using a 5:1 flask:culture volume ratio for good aeration. For larger preparations, 1ml of overnight culture was further grown in larger (250ml) volumes of LB broth. Optical density of 0.8-1.0 was targeted as optimal for log phase growth. Bacteria were centrifuged at 8,000*g* for 2 minutes at room temperature. Pellets were resuspended in glucose buffer (buffer I section 2.12.1), using $1/10^{th}$ volume of culture=1 volume) by vigorous vortexing/pipetting. Bacteria were then lysed using 2 volumes of lysis buffer II (section 2.12.1), and rolled until a viscous lysate was formed. 3 volumes of cold high salt buffer (buffer III, section 2.12.1) were then added and the samples mixed until a genomic DNA/SDS/protein precipitate formed. Samples were chilled on ice for 10 minutes to maximise genomic DNA yield, and then centrifuged at 16,110*g* for 20 minutes to ensure protein precipitation. The genomic DNA replete supernatant was aspirated and filtered to

ensure a clean bacterial lysate yield. For small 2ml (mini-) preps, phenol/chloroform extraction was performed: an equal volume of phenol/chloroform was added to the cleared bacterial lysate before vortexing and centrifugation at 16,110*g*. The aqueous supernatant was transferred to a fresh eppendorf tube and the remaining phenol/chloroform discarded. This was repeated once, 450µl of room temperature propan-2-ol (isopropyl alcohol, IPA) added and mixed, and the sample centrifuged at 16,110*g* for 10 minutes. Supernatant was aspirated and the DNA pellet air-dried for 5 minutes before re-suspension in 50µl TE. To minimise RNA contamination, RNAse treatment was performed prior to phenol/chloroform extraction. For larger plasmid preparations, IPA ($1/7^{th}$ volume of bacterial lysate) was added to precipitate nucleic acid, which was further separated by centrifugation (16,110*g* for 10 minutes, or until supernatant cleared). Supernatant was then aspirated and the pellet dried, before re-suspending in $1/50^{th}$ volume dH_2O. RNA was precipitated by adding an equal volume of LiCl solution chilling on ice for 30 minutes and centrifuging at 16,110*g* for 10 minutes). Supernatant was transferred to a fresh tube and an equal volume of phenol/chloroform added, and the mixture centrifuged at 3,000*g* for 4 minutes. The aqueous layer was transferred to a fresh tube, and phenol/chloroform discarded. This was repeated once, and then DNA precipitated in 2.2 volumes of cold ethanol (sample chilled at -20°C for one hour). DNA was then pelleted by centrifugation at 16,110*g* for 10 minutes and all supernatant removed. The pellet was then RNAse treated (resuspended in 200ml (for initial culture volumes of 200-500ml) or 2ml (initial culture volumes of 500ml and above) of TE/RNAse (20µg/ml), and incubated for one hour at 37°C. Phenol/chloroform extraction was again performed, and DNA precipitated with $1/10^{th}$ volume sodium acetate, and then 2.2 volumes cold ethanol. Purified plasmid DNA was centrifuged at 16,110*g* /10 mins, ethanol removed and the pellet re-suspended in 100µl (medium preps) or 1ml (large preps) of TE.

2.13 RNA preparation

Cells grown in monolayers were lysed directly by the addition of 1ml TRIzol per 10^6 cells, and repeatedly pipetting to ensure homogenisation. Cells in suspension were pelleted by centrifugation in 14mL polypropylene Falcon tubes, and liquid supernatant discarded. 1mL of TRIzol (Gibco Life Sciences Paisley) was added per 10^6 cells. Cells were disrupted by shaking for 1 minute room temperature (RT), and then allowed to stand for 5 minutes at RT to allow dissociation of nucleoprotein complexes. 0.2mL chloroform was then added for each 1mL of TRIzol used. The mixture was shaken vigorously by hand for 15 seconds and allowed to stand at RT for a further 2 minutes. Samples were then centrifuged for 10 minutes at 10,000*g* at 4°C, ensuring clear separation into an upper aqueous (RNA) phase, an

interphase (DNA) and a lower red phenol/chloroform (organic protein and contaminant) phase. The top aqueous RNA rich layer was transferred and aliquoted into RNase-free 1.5ml Eppendorf tubes, and then precipitated with 0.5mL 100% isopropyl alcohol added per 1mL TRIzol used. Samples were allowed to rest for 10 minutes at RT before inverting 3 times to mix, and centrifuging for 15 minutes at 10,000*g* at 4°C. The supernatant was carefully poured off, and the residual "glassy" or gel-like RNA pellet or smear became apparent at the base or wall of the tube. The pellet was washed 1mL 75% ethanol per ml if TRIzol originally used, vortexed and then centrifuged at 7,500g at 4°C for 5 minutes. The ethanol wash was repeated to remove salt from the RNA pellet. The pellet was then briefly air-dried at RT for 5 minutes, before re-suspending it in 100ml RNase-free water and assessing purity by spectrophotometry at 260nm and 280nm. An A_{260}/A_{280} absorption ratio of >1.8 was deemed minimum purity, and ribosomal RNA integrity was further checked by gel electrophoresis. RNA was utilised immediately or stored at -80°C.

2.14 DNA extraction following gel electrophoresis

Gel extractions were performed on gel purified restriction fragments prior to ligations, and on ligation products prior to transformation of competent bacteria. Qiagen gel extraction kits were used. In brief, DNA fragments were excised from TAE or TBE/agarose gels using a sharp scalpel under UV light guidance. Gel slices were transferred to 1.5ml Eppendorf containers and weighed. Solubilising buffer (Buffer QG, Qiagen UK) was added to gel slices, using 3 volumes of buffer for each unit gel mass (e.g. 300µl for 100µg gel), and incubated for 10 minutes at 50°C. Solubilisation was assisted by intermittent vortexing of gel fragments. pH was adjusted to 5.0 using a commercial pH indicator (Qiagen). DNA was then bound to a proprietary membrane in a centrifugation column (QIAquick spin column, Qiagen, UK), with the sample being centrifuged at 16,110*g* for 1 minute. To remove agarose, a further treatment of dissolving buffer was added and the sample centrifuged, before DNA treatment with wash buffer, and final centrifugation at 16,110*g* for 1 minute. DNA was then eluted by adding 50µl of elution buffer (10mM TrisHCl, pH8.5) or nuclease free water to the DNA binding membrane and the column re-centrifuged at 16,110*g* for 1 minute.

2.15 Transfection of THP-1 cells with mutant PPARγ DNA

Transfection of THP-1 cells was performed using electroporation techniques. Equibio electroporators or Amaxa nucelofector electroporators were used, with pulse strength varied to optimise transfection success. THP-1 cells were split 1:2 in Iscoves medium with serum to ensure log phase growth at the time of transfection. Electroporation was performed in 4mm

cuvettes, using 10μg of DNA per $1x10^6$ cells. Both single and double pulse protocols were used. Cell membrane permeability was assessed by trypan blue exclusion immediately after electroporation, and cell viability of less than 5% was aimed for. As an alternative to Equibio protocols, transfection was attempted in nocodazole, a cell cycle inhibitor. Cells were held in G_0 by pre-treatment with nocodazole to promote DNA entry during electroporation. Cell viability was assessed by trypan blue exclusion and transfection success by flow cytometry and fluorescence microscopy at 488nm.

2.16 Measurement of intracellular calcium levels

1.5ml aliquots of monocytes pre-treated with $15dPGJ_2$ were equilibrated in a Perkins Elmer spectrometer for 10mins, or until a stable baseline was achieved. The spectrometer was set at excitation wavelengths of 340 and 380nm, with an emission wavelength of 510nm. Following equilibration, a baseline was recorded for 1 min prior to the addition of platelet activating factor, fMLP and leukotriene B4 as positive control stimuli, and digitonin to permeabilise the cell membrane. Absolute calcium levels were not calculated.

Comments for pcDNA3:
5446 nucleotides

CMV promoter: bases 209-863
T7 promoter: bases 864-882
Polylinker: bases 889-994
Sp6 promoter: bases 999-1016
BGH poly A: bases 1018-1249
SV40 promoter: bases 1790-2115
SV40 origin of replication: bases 1984-2069
Neomycin ORF: bases 2151-2945
SV40 poly A: bases 3000-3372
ColE1 origin: bases 3632-4305
Ampicillin ORF: bases 4450-5310

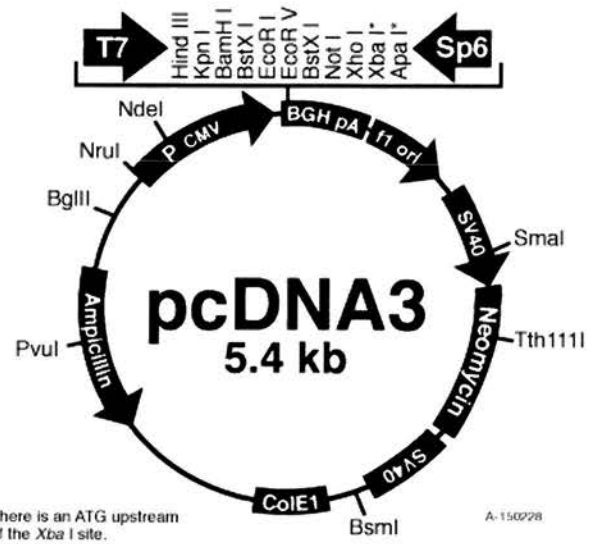


Figure 2-1 pcDNA3

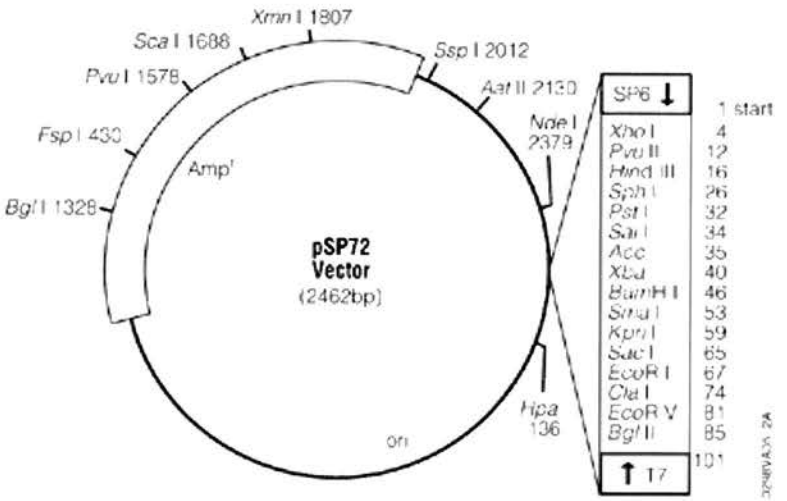
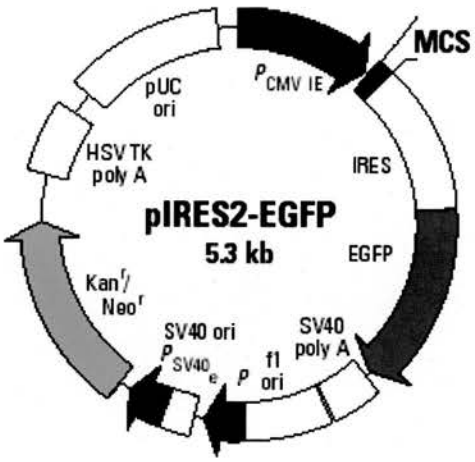


Figure 2-2 pSP72



591 601 611 621 631 641 651 661

GCTAGCGCTACCGGACTCAGATCTCGAGCTCAAGCTTCGAATTCTGCAGTCGACGGTACCGCGGGCCCGGGATCC...IRES

Nhe I Eco47 III Bgl II Xho I Sac I Ecl136 II Hind III EcoR I Pst I Sal I Acc I Kpn I Asp718 I Apa I Bsp120 I Sac II Xma I Sma I BamH I

Figure 2-3 pIRES2-GFP

Chapter 3 MONOCYTE APOPTOSIS INDUCED BY MODIFIED LOW DENSITY LIPOPROTEINS AND CYCLOPENTENONE PROSTAGLANDINS

3.1 Introduction: impact of apoptosis on inflammation

Apoptotic cells have the potential to influence the outcomes of local inflammatory reactions (Haslett et al. 1994). Infiltrating leukocytes, usually granulocytes, undergo programmed cell death once their actions at tissue injury or infection sites are complete, and inflammatory stimuli have been removed (Grigg et al. 1991). Monocyte recruitment to an area of inflammation follows granulocyte recruitment (Hopper 1986) perhaps reflecting monocyte roles in the clearance of effete granulocytes and cellular debris, and the regulation of tissue repair. Mature macrophages specifically recognise apoptotic cells, *via* mechanisms that allow non-inflammatory clearance to occur with minimal tissue injury (Fadok et al. 1998b). By contrast, necrotic cell debris initiates pro-inflammatory macrophage behaviour, resulting in a more aggravated local cellular response, with the production of pro-inflammatory cytokines, secretion of proteolytic enzymes and resultant local extracellular matrix degradation (Savill & Haslett 1995). This has implications for the optimal resolution of inflammatory insults. A defined time period for non-inflammatory clearance and repair exists, beyond which pro-inflammatory mechanisms are initiated (Savill et al. 2002). Macrophage responses to apoptotic cells vary temporally, with initial priming of inflammatory effector mechanisms being followed by the secretion of the anti-inflammatory cytokine TGF-β (Lucas et al. 2003).

3.1.1 Apoptotic effector mechanisms

Apoptosis represents a mechanism of cell deletion that has been conserved through evolution, with caspase group of cysteine proteases intrinsic to this process. Two caspase-dependent pathways leading to apoptosis, termed extrinsic and intrinsic (Scoltock & Cidlowski 2004). The extrinsic or death receptor pathway is initiated by TNF-receptor family members by recruiting adaptor and signalling molecules to assemble the death-inducing signalling complex (DISC) (Kischkel et al. 1995). This complex leads to activation of caspases 8 and/or 10, which then activate downstream effector caspases such as caspase 3, 6, and 7 (Medema et al. 1997). An alternate extrinsic pathway operates in so-called Fas-responsive type II cells. Plasma membrane receptors signal to the mitochondria allowing caspase 8 to cleave Bid. The truncated form of Bid (tBid) translocates to the mitochondrial membrane, triggering apoptosis *via* mitochondrial release of cytochrome c and downstream caspase 9 activation (Li et al. 1998; Scaffidi et al. 1998).

An intrinsic pathway for apoptosis is activated by cellular stress stimuli, including serum deprivation. The mitochondrion undergoes loss of membrane potential ($\Delta\Psi_{mito}$) following the insertion of pro-apoptotic Bax sub-family proteins into the membrane. Apoptotic proteins, particularly cytochrome *c*, are then released, activating formation of the apoptosome (activated caspase 9 and apoptotic protease activating factor 1, or Apaf-1), a process regulated by Bcl-2 family members (Kroemer, Zamzami, & Susin 1997). Further regulation of caspases occurs beyond mitochondrial checkpoints, with inhibitors of apoptosis proteins (IAP) binding and inhibiting caspase 9 (Devereux 1999) and possibly caspase 3. IAPs are themselves regulated by Smac/Diablo, (Chai et al. 2000), with this pathway eventually converging on downstream effectors of cell death including caspase 3 (reviewed by Green 1998). The role of caspases in dissecting apoptosis is thus of major interest, and offers insights into potential routes of manipulating cell death.

Cell viability is dependent upon a balance between normal cell cycle progression and factors that induce cell-death. Tumour suppressor genes including p53 and the retinoblastoma (Rb) gene regulate inappropriate cell cycle progression and are central to this balance. Such anti-oncogenes promote apoptosis, limiting neoplasia (Evan & Littlewood 1998). Cell cycle checkpoints have been extensively explored in the context of VSMC apoptosis (Mercer et al. 2005), and in macrophages (van Vlijmen et al. 2001), but were not examined in these studies. Monocyte apoptosis has been reviewed in Chapter 1, but the implications of apoptosis for inflammatory vascular injury merit further description.

3.1.2 Imbalances in apoptosis & clearance govern local inflammation

Resolution of inflammatory injury requires efficient recognition ingestion and disposal of apoptotic debris by professional phagocytes (Savill & Fadok 2000). Physiologically appropriate clearance of apoptotic cells engenders anti-inflammatory macrophage responses including suppression of TNF-α (Voll et al. 1997), and promotion of TGF-β secretion in specific recognition of cell membrane phosphatidylserine (PS) exposure (Huynh, Fadok, & Henson 2002) aiding local tissue repair. By contrast, inefficient phagocytic clearance impairs local repair and resolution of tissue injury, a phenomenon definitively proven in targeted gene deletion models of the C1q complement component which acts as a phagocytic receptor (Botto et al. 1998). Reduced apoptotic clearance is seen in clinical systemic lupus erythematosus (SLE) and promotes local inflammation (Herrmann et al. 1998). Uncleared apoptotic cells progressing to secondary necrosis produce further adverse sequelae. Monocyte-derived dendritic cells invoke pro-inflammatory responses from T cells following the ingestion of necrotic but not apoptotic cells (Sauter et al. 2000). Two alternative

outcomes follow augmented local inflammation. Fibroblastic responses may predominate, with loss of functional tissue. Models of inflammation using MRL/Mp mice prone to systemic lupus erythematosus show alterations in TGF-β secretion reflecting the fibrosis seen in clinical SLE (Kench et al. 1999). Alternatively, continuing inflammation may cause granuloma formation as seen in mycobacterial infection. This may be driven by over-expression of osteopontin, mediating elevated macrophage influx, and increasing local IL-8 and TNFα secretion (Nau et al. 1997). Osteopontin may mediate calcification in advanced atherosclerotic lesions, reflecting generic mechanisms that operate during chronic inflammation (Abedin, Tintut, & Demer 2004). An additional important consideration is the effect of ingestion of apoptotic cells upon antigen cross-presentation, a task that is under-taken by immature monocyte-derived dendritic cells in a discrete integrin mediated fashion (Albert et al. 1998). Although apoptotic cells are cleared in a non-inflammatory manner, dendritic cells are able to process apoptotic cell contents and efficiently present them to CD-8+ T-lymphocytes in a manner that may promote auto-immunity (Albert, Sauter, & Bhardwaj 1998). Responses to apoptotic cell membranes as well as cell contents may thus be a factor in promoting local atherosclerotic inflammation, and provide a further argument for an auto-immune component to atherogenesis.

3.1.3 Inflammatory imbalances relevant to vascular injury

Vascular inflammation and in particular, the effects of endothelial injury are of particular importance in the recruitment of platelets and leukocytes to the surface of damaged vessel lumina, as discussed in Chapter 1 section 1.7. Local apoptosis of endothelial cells promotes thrombogenesis (Bombeli et al. 1997). Pro-thrombotic factors not only regulate platelet and haemostatic function, but also direct inflammatory responses in the vessel wall (Major et al. 2003). Platelet sequestration to endothelial surfaces necessitates phagocytic clearance: macrophages are less susceptible to apoptosis following ingestion of platelets (Lang et al. 2002), prolonging the life of such macrophages at sites of vascular injury. Activated macrophages may induce Fas-mediated apoptosis in neighbouring VSMCs, with adverse consequences for atherosclerotic plaque structural stability (Boyle et al. 2001). Apoptotic VSMCs generate thrombin (Flynn et al. 1997), stabilising local thrombus by cleaving fibrinogen to fibrin, and activating platelets (Davey 1967). Monocytes may display paradoxical activation by pro-apoptotic stimuli such as Fas ligand ligation (Park et al. 2003), demanding careful exploration of any monocyte viability responses to microenvironment.

Efficient apoptotic cell clearance may be compromised in atherosclerosis, resulting in local phagocytic capacity being exceeded (see section 3.1.2). This phenomenon that may be

directly linked to apoprotein E levels the absence of which has been associated with enhanced levels of systemic macrophage apoptosis as well as reduced clearance (Grainger, Reckless, & McKilligin 2004). Apoptotic control mechanisms, especially in the context of impaired clearance and progression to necrosis, are thus of direct interest in the study of atherosclerosis.

3.1.4 Evidence of apoptosis in atheroma

Multiple studies have established the presence of apoptotic cells in atherosclerotic plaque . Variation in reported levels of apoptosis reflect diverse methodologies used in quantification. Human fatty streaks contain pro-apoptotic proteins, in contrast to areas of adaptive intimal thickening. High levels of apoptosis have been shown in advanced atherosclerotic plaques with dense macrophage infiltration (Kockx 1998). Late apoptotic macrophages are more highly represented in areas of atherosclerotic lesions surrounding the necrotic core. Human ruptured plaques show extensive macrophage infiltration of plaque fibrous caps. Active apoptosis, defined by the presence of the apoptotic precursor caspase-1, is seen in areas of plaque degradation and macrophage infiltration (Kolodgie et al. 2000). This argues strongly for a role of monocyte apoptosis in plaque stability.

3.1.5 Pro-atherogenic factors that promote apoptosis

Multiple pro-apoptotic stimuli exist in atherosclerotic plaques, where VSMCs and ECs as well as monocyte/macrophages may be exposed to biologically active micro-environmental factors that modulate cell death. These include mechanical shear forces (Tricot et al. 2000), reactive oxygen species (Heermeier et al. 1999), and pro-inflammatory T-lymphocyte derived cytokines (Geng et al. 1996) as well as lipid species (Galle et al. 1999), resulting in a dynamic turnover of cells within active plaque. In particular, the pro-apoptotic roles of high levels of nitric oxide (Patel et al. 2000) and local hypoxia (Aoki et al. 2001) present within atherosclerotic plaque have been highlighted. These factors together with other detrimental stimuli such as altered local levels of growth factors that would normally promote survival, all contribute to a milieu that enhances apoptosis of vulnerable cells.

Lipid accumulation is a separate pro-apoptotic stimulus, with free cholesterol (FC) loading of macrophages being linked to cell death (Tabas 2002), possibly because of defective cholesterol efflux caused by dysfunctional ABCA-1 transport mechanisms (Feng & Tabas 2002). Excessive intra-cellular FC may contribute to alterations in the physical compliance of the endoplasmic reticulum ER. This alters integral ER membrane protein mobility, affecting downstream signalling pathways including sarcoendoplasmic reticulum ATPase (SERCA). Activation of the ER-based Unfolded Protein Response (UPR) signal transduction

pathway results in the activation of the C/EBP homologous protein (CHOP) (Zinszner et al. 1998). Downstream death pathways are then activated in cholesterol laden macrophages (Feng et al. 2003), including c-Jun NH2-terminal kinase (JNK)2, in a manner that is directed by SR-A ligation by lipids (DeVries-Seimon et al. 2005). The mechanics of cholesterol-induced apoptosis are complex. Early studies suggest that free cholesterol promotes mitochondrial stability (Graham & Green 1970), arguing for mitochondrial independent apoptotic paths. Mitochondrial control of apoptosis is often pivotal in directing leukocyte cell death, activating signalling cascades including the Smac/Diablo pathway, and the elaboration of initiator caspases (Maianski et al. 2004). However, further data suggest that the modification state of LDL may be more important in dictating apoptotic responses.

3.1.6 Ox-LDL and atheroma: links to apoptosis and leukocyte responses

Oxysterols and ox-LDL have been linked to apoptosis in atherosclerotic lesions (Salvayre et al. 2002). Discrepancies exist in the literature between the correlation of ox-LDL and macrophage-rich areas in plaque (Akishima et al. 2005), with some reports that ox-LDL occupies medial territory deep within the vessel wall beyond sites of active macrophage infiltration (Fukuchi et al. 2002). However, oxysterol species have been found to be highly represented within the necrotic core of atherosclerotic plaques (Garcia-Cruset et al. 1999), an area where the lipid pool and cell debris are products of the programmed cell death of local plaque cell populations (see Chapter 1, sections 1.3 and 1.17). Ox-LDL appears to differentially augment Fas ligand expression in endothelial cells that have undergone hypoxia/re-oxygenation injury, suggesting that local ox-LDL may adversely impact upon cell survival in atherosclerotic plaque (Li, Yang, & Mehta 1998).

3.1.7 Cyclopentenone induction of apoptosis

Prostaglandins (PGs) are a family of naturally occurring cyclic 20-carbon fatty acids synthesised mainly from arachidonate and other fatty acids released from cell membrane phospholipids by phospholipases (Straus & Glass 2001). Arachidonic acid is derived from the action of phospholipase A_2 upon membrane phospholipids, and is thus present at sites of inflammation, where leukocytes undergo cell death (Zurier 1993). Cyclooxygenase (COX) transforms arachidonic acid into an unstable endoperoxide intermediate, which is converted by PG synthases into PGD_2, PGE_2, and $PGF_{2\alpha}$. Prostaglandins of the J series originate from dehydration within the cyclopentane ring of PGD, producing a cyclopentenone structure characterized by the presence of a reactive α,β-unsaturated carbonyl group.

Cyclopentenone prostaglandins (cyPGs) are biologically very potent molecules with diverse actions, and act as natural ligands for the nuclear steroid hormone receptor PPARγ, (reviewed in Chapter 1, section 1.19). Their influence upon the cell cycle is marked, with the ability to induce cell growth arrest (Santoro et al. 1986). The terminal derivative of prostaglandin J_2 (PGJ_2) metabolism, 15-deoxy-$\Delta^{12,14}$-PGJ_2 ($15dPGJ_2$), shows particularly marked effects upon cell survival. $15dPGJ_2$ has been reported to induce cell death *in vitro* and *in vivo* in tumour cells. Autophagic cell death is induced by $15dPGJ_2$ in prostatic tumour cells (Butler et al. 2000), whereas classical apoptosis is induced by $15dPGJ_2$ in tumour cells from mammary (Clay et al. 1999), colonic (Shimada et al. 2002), haematopoietic (Laurora et al. 2003), and lymphoid malignancies (Padilla, Leung, & Phipps 2002). cyPGs also display anti-inflammatory activity, an observation that has been directly linked to the induction of granulocyte and macrophage apoptosis (Gilroy et al. 2003).

3.1.8 Role of NF-κB in cyclopentenone effects upon cell survival

15dPGJ2 was shown to exert some of its effects by binding to the peroxisome proliferator-activated receptor γ (PPARγ) (Kliewer et al. 1995). However, although 15dPGJ2 induces classical changes in mitochondrial potential during lymphocyte apoptosis, this may not occur in an exclusively PPARγ specific manner (Nencioni et al. 2002). Further evidence suggests that $15dPGJ_2$ may act independently from PPARγ activation, with disparities between cyPGs and thiazolidinedione PPARγ ligands being evident in assays of cell function (Boyault et al. 2001) as well as survival (Ward et al. 2002). $15dPGJ_2$ acts as a potent inhibitor of the I-κB kinase (IKK), preventing the activation of nuclear factor-κB (NF-κB) through PPARγ-independent means, offering an alternative mechanism for apoptotic induction by cyPGs (Rossi et al. 2000). Given that NF-κB is a critical regulator of early inflammatory responses, as well as cell proliferation and survival, the latter consideration is important. Normally an inactive intra-cytoplasmic complex, NF-κB is composed of a heterodimer of p50 and p65 (RelA) subunits bound to inhibitory proteins of the I-κB family (I-κBs). NF-κB is induced in response to a range of stimuli varying from UV radiation, pro-inflammatory cytokines or mitogens and microbial infection, including bacterial LPS (Li & Verma 2002). NF-κB induction by pro-inflammatory cytokines requires activation of the IKK complex, containing two catalytic subunits, IKKα and IKKβ, and the IKKγ regulatory subunit. The complex phosphorylates I-κBs triggering their ubiquitination and proteasome-mediated degradation (Karin & Ben Neriah 2000). I-κB release permits nuclear translocation of NF-κB and its binding to DNA at specific κB sites, inducing the transcription of genes encoding cell

adhesion molecules, inflammatory and chemotactic cytokines, cytokine receptors, and enzymes that produce inflammatory mediators (Li & Verma 2002). Studies show that NF-κB activation protects cells from apoptotic pathways that might be invoked during inflammation, including TNFα mediated cell death (Beg & Baltimore 1996). Inhibition of NF-κB by $15dPGJ_2$ in Burkitt lymphoma cell lines induces the down-regulation of the expression of several NF-κB-dependent anti-apoptotic gene products and the activation of caspase-8 and caspase-9 (Piva et al. 2005). Evidence suggests that PPARγ may be similarly irrelevant to inflammatory leukocyte apoptosis, the survival of which is more clearly governed by I-κB degradation (Ward et al. 2002). Exploration of the role of cyPGs was thus of interest not only in evaluating the survival responses of monocytes entering a pro-inflammatory environment but also in order to assess the biochemical mechanism behind any alterations in survival that might be evident.

3.1.9 Link between ox-LDL and cyclopentenones *via* PPARγ

Potential links between PPARγ and ox-LDL were introduced in Chapter 1. PPARγ plays a role in regulating lipid flux in monocytes (Chawla et al. 2001b), the complexity of which was previously underestimated (Evans 1988). Critical inflammatory functions are induced by PPARγ ligation with modified LDL products. Altered phosphocholines, acting as natural PPARγ ligands to enhance endothelial cell production of MCP-1 and IL-8 (Lee et al. 2000). Given the data showing that free cholesterol accumulation drives apoptosis (Tabas 2000), the effects of PPARγ upon CD36 expression suggest a potential paradox. The concept of unlimited lipid influx should lead to monocyte cell death, yet certain macrophages remain viable in atherosclerotic plaque, despite an adverse local environment. Monocyte/macrophages have been shown to undergo cyPG-induced apoptosis in a manner ascribed to both PPARγ and PPARα mediated mechanisms (Chinetti et al. 1998). If this were true, PPARγ-mediated apoptosis should modulate monocyte survival in response to modified LDL. A pro-apoptotic response might further alter cell numbers in an inflamed atherosclerotic plaque with a high local concentration of cyPGs. Monocyte survival responses to PPARγ ligation are relevant to the use of high-affinity ligands of the thiazolidinedione family, which act to alter insulin resistance at a cellular level. These agents have been shown to be anti-inflammatory (Ricote et al. 1998b), and may act to stabilise atherosclerotic plaque (Li et al. 2000). Elucidating a role, if any, for PPARγ in monocyte survival is thus of central interest in determining monocyte cell population changes in atherosclerotic plaque.

3.1.10 Aims and objectives in assessing monocyte survival responses

The aims of this chapter are to discern if LDL induces monocyte apoptosis, and whether LDL oxidation state influences monocyte survival.

Specifically:

- The involvement of PPARγ in ox-LDL-mediated effects upon monocyte survival will be assessed.
- The survival of cells bound to different extracellular matrices will be measured to assess a regulatory role of adhesion upon monocyte apoptosis.
- The biochemical mechanisms governing monocyte activation and survival will be explored.

3.2 Results

All data tables and photomicrographs referred to are viewable on the supplementary CD-ROM, and in print form in the appendix.

3.2.1 Monocyte preparation and purification

Peripheral blood monocytes were isolated using discontinuous Percoll™ gradient centrifugation, and negative immuno-selection using magnetic bead separation. Purities of >90% (as assessed by flow cytometry scatter profiles) were achieved with this technique. Data for this are presented in Chapter 6, (section 6.2.2), as the same methods were used to isolate cells for monocyte phenotyping experiments. Monocytes were assessed for apoptosis in suspension culture and adhesion culture. Cells in suspension appeared to reduce in cell number with prolonged periods of culture with less than 40% recovery after 24hrs (n=5, see Chapter 3 appendix, Table 3.1). Furthermore, it was apparent that cell fragments and post-apoptotic cell bodies were not visible in cell suspensions (data not shown). This suggested that fragile late apoptotic cells and apoptotic bodies were not being assessed. For this reason, further assessments of apoptosis were performed on cells in adherent culture, using morphological changes to quantify apoptosis. Although DNA intercalating dyes label nuclear material, it was also possible to visualise apoptotic bodies using this method.

3.2.2 Oxidised LDL induces monocyte apoptosis

Monocytes were exposed to control serum-free medium, or to ox-LDL at 50μg/ml, a concentration that is physiologically active and has been used to produce a monocyte-derived foam-cell phenotype *in vitro* (Nagy et al. 1998), for 24hrs. Control cells retained monocyte nuclear architecture with a typical horseshoe appearance, and open granular nuclear matrices (Figure 3.1). Ox-LDL exposed monocytes displayed nuclear morphological changes consistent with apoptosis. Apoptotic cells showed punctate nuclei consistent with chromatin condensation caused by DNA damage in the execution phase of apoptosis (figure 3.2). Further evidence that monocyte were undergoing apoptosis was provided by assessing Annexin V-FITC binding on ox-LDL exposed monocytes in suspension. This confirmed that ox-LDL exposure induced monocyte PS exposure (Figure 3.3). Further assessments of apoptosis were made by morphological criteria alone.

3.2.3 Oxidised LDL induced monocyte apoptosis is concentration dependent

The level of oxidised low-density lipoprotein (ox-LDL) necessary for monocyte apoptosis was determined by examining concentration responses of adherent cells exposed in serum-

free conditions for 24 hours to increasing concentrations of ox-LDL. In these experiments a concentration-dependent relationship between monocyte apoptosis and ox-LDL levels was observed when compared to control untreated monocytes (Table 3.2, Figure 3.4). Although untreated monocytes in serum-free culture displayed constitutive apoptosis, in the presence of ox-LDL the observed percentage of cell death was significantly elevated (2-way ANOVA, $p<0.0001$, $n=4$). This was seen for all concentrations between 25μg/ml and 100μg/ml. A significant reduction in apoptosis was seen for all ox-LDL concentrations with the addition of serum to the culture medium (2-way ANOVA, $p<0.0001$, $n=4$). It is speculative to predict the levels of oxidised LDL components that might be present within active atherosclerotic lesions, making direct comparisons of these *in vitro* LDL levels with concentrations found *in vivo* difficult. Local levels of such lipid species may be concentrated to a high level within active lesions. For the purpose of these experiments, it was important to use a concentration of ox-LDL capable of consistently inducing apoptosis. For this reason the concentration of 50μg/ml was used for subsequent experiments.

3.2.4 LDL induction of apoptosis is dependent on oxidation status

To assess whether specific alterations of LDL were important in the induction of monocyte apoptosis in serum-free culture, a direct comparison of the effects of a 50μg/ml level of ox-LDL with the same concentration of native LDL was made. Ox-LDL oxidation status was assessed by electrophoretic mobility, which was qualitatively shown to be higher than n-LDL mobility (Appendix Figure 3.1). Monocyte apoptosis was not altered by n-LDL, but was significantly increased by exposure to 50μg/ml ox-LDL (Friedman one-way ANOVA, $n=4$, $p=0.0046$). (Table 3.3, Figure 3.5)

3.2.5 Effect of caspase inhibition upon ox-LDL induced apoptosis

The dependence of ox-LDL induced apoptosis upon caspases, apoptotic execution enzymes, was assessed. Purified naïve monocytes in serum-free culture were pre-treated with 100μM of the pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone (zVAD-fmk) for 30 mins before exposure to oxidised LDL at 50μg/ml and 100μg/ml. Although a decrement in apoptosis was seen in zVAD-fmk treated samples, inhibition of apoptosis was incomplete (2-way ANOVA, $n=4$, $p=0.7289$) (Table 3.4, Figure 3.6).

3.2.6 Effect of a PPARγ antagonist upon ox-LDL-induced monocyte apoptosis

To ascertain whether PPARγ-induced mechanisms mediated ox-LDL-induced monocyte death, monocytes were pre-incubated with 10μM of GW9662, a specific pharmacological antagonist to PPARγ (see section 3.2.16). No significant inhibition of ox-LDL mediated death was apparent (2-way ANOVA with Bonferroni post-tests, p=0.3532, n=4, see Table 3.5, Figure 3.7). This observation suggested that ox-LDL induced apoptosis might be mediated *via* a PPARγ independent mechanism.

3.2.7 Ox-LDL products that are natural ligands for PPARγ produce low level monocyte apoptosis

13-*S*-hydroxyoctadecadienoic acid (13(*S*)HODE) is the main product of 15-lipoxygenase-1 (15-LOX-1) metabolism of linoleic acid (Zuo et al. 2005). Previous workers in this field have used concentrations of 10μM or up to 10μg/ml in assaying the functional activation of PPARγ and consequent downstream sequelae (Bull et al. 2003). The effects upon monocyte apoptosis of a range of concentrations of 13(S)HODE were tested and compared against control media and the vehicle (DMSO) in which the 13(S)HODE was solubilised. Adherent and suspension cultured monocytes were treated for 24 hours in serum-free conditions. Low levels of 13(S)HODE induced no statistically significant increment over constitutive apoptosis in adherent monocyte culture (2-way ANOVA, n=4, p=0.0217) (see Table 3.6, Figure 3.8).

3.2.8 High-affinity synthetic PPARγ ligands do not induce monocyte apoptosis

To address the role of PPARγ in the induction of monocyte apoptosis adherent monocytes were treated with ciglitazone, a thiazolidinedione class of PPARγ agonists. Published data suggest that this synthetic PPARγ ligand binds and activates the receptor nanomolar concentrations (Gurnell et al. 2000). A saturating concentration may be achieved with these ligands at between 1 and 10μM during *in vitro* culture (Tontonoz, Hu, & Spiegelman 1995). A range of ciglitazone concentrations was tested upon monocyte survival responses. These experiments showed evidence of significant elevation of apoptosis rates for ciglitazone at 1μM, (2-way ANOVA, n=4, p=0.0217) but not for other concentrations of ciglitazone (2-way ANOVA, n=4, p=0.0217) (Table 3.7, Figure 3.9).

3.2.9 Cyclopentenone prostaglandins induce monocyte apoptosis in serum-free conditions

The effects of the cyPGs Δ12PGJ$_2$ and 15dPGJ$_2$ were examined to assess the role of these inflammatory mediators and naturally occurring PPARγ ligands on primary monocyte survival. Initial studies showed that 1μM concentrations of cyPG did not elevate monocyte apoptosis. 10μM concentrations, reported as physiologically active, and 100μM concentrations of Δ12PGJ$_2$ and 15dPGJ$_2$ induced similar and significant levels of monocyte apoptosis (n=4, $p<0.0001$, Table 3.8, Figure 3.10). Further studies confirmed that monocyte apoptosis was significantly enhanced by 10μM Δ12PGJ$_2$ and 15dPGJ$_2$, in a manner dependent upon serum-withdrawal (2-way ANOVA, $p<0.0001$, n=10) (Table 3.8, Figure 3.11). In parallel experiments, THP-1 monocytic cells were exposed to the 10μM of 15dPGJ$_2$ and cultured in two different types of serum-free media. THP-1 cells demonstrated a significant apoptotic response to 15dPGJ$_2$ (2-way ANOVA, $p<0.0001$, n=4, Table 3.9, Figure 3.12), but with lower absolute levels of cell death in comparison to primary human monocytes. cyPG-induced apoptosis was significantly reduced by the pan-caspase inhibitor zVAD-fmk, (2-way ANOVA, $p<0.001$, n=4), although this did not return apoptosis rates to control levels (Table 3.10, Figure 3.13).

3.2.10 Cis-retinoic acid fails to induce further augmentation of monocyte apoptosis in conjunction with cyclopentenones

Ligation of PPARγ is the first biochemical step in altering transcription *via* this nuclear hormone super-family member. PPARγ forms a heterodimer with RXR, resulting in a complex that binds onto a PPAR response element (PPRE), an upstream binding sequence at the 5' end of target genes (Tugwood et al. 1992). The activity of RXR thus introduces a further level of control of gene transcription PPAR-mediated nuclear signalling. However it is also possible for 9 (cis) retinoic acid and other synthetic RXR ligands to act independently at this site (Kliewer et al. 1992) owing to the permissive nature of the RXR component of the heterodimer site (Mangelsdorf & Evans 1995). One prediction would be that RXR ligands such as cis-retinoic acid would act to augment monocyte apoptosis, if it was driven by a PPARγ/PPRE mediated mechanism. To assess the validity of this suggestion, 9 cis-retinoic acid was used to co-treat adherent monocytes in serum-free culture. For each individual treatment, no significant further up-regulation of apoptosis was seen with 9 (cis) retinoic acid (2-way ANOVA, $p=0.2116$, n=4) (Table 3. 11, Figure 3.14).

3.2.11 PPARγ antagonism does not limit cyclopentenone induced monocyte apoptosis

The action of cyPGs has been suggested to modulate the transcription of genes central to inflammatory activation, by acting *via* PPARγ to induce anti-inflammatory phenotypes in a variety of leukocytes with resultant beneficial effects on resolving tissue injury (Willoughby, Moore, & Colville-Nash 2000). Evidence from luciferase reporter-sequences indicates that PPARγ activation may block NF-κB activation albeit at high concentrations (Rossi et al. 2000). This suggests that modulation of anti-inflammatory activity is achieved at a transcriptional level, and might affect leukocyte apoptosis. The effects of pharmacological PPARγ blockade upon cyPG-induced monocyte cell death were tested. Monocytes cultured in serum-free conditions were pre-treated with 10μM of the specific PPARγ antagonist GW 9662 prior to exposure to cyPGs. No significant reduction in cyPG-induced apoptosis was seen (2-way ANOVA, n=3, p=0.7053) (Table 3.12, Figure 3.15).

3.2.12 Factors modulating cyclopentenone-induced monocyte apoptosis: maturation

Maturation may offer protection against apoptosis, a phenomenon noted in monocyte-derived DCs (Lundqvist et al. 2002). To examine if this was relevant to cyPG-induced monocyte apoptosis, adherent monocytes were matured for increasing periods of time before exposure to cyPGs. Monocytes in medium supplemented with 10% autologous serum for 24hrs, 48hrs and 6 days were washed in serum-free PBS, prior to a further 24-hour period of culture in serum-free conditions with or without cyPGs. Monocyte apoptosis was significantly reduced in all pre-cultured cells (2-way ANOVA, $p<0.0001$, n=3), in a manner that appeared to relate to their maturation state (Table 3.13, Figure 3.16).

3.2.13 Factors modulating cyclopentenone-induced monocyte apoptosis: adhesion

To investigate whether monocyte adhesion to matrices regulated monocyte apoptosis, monocytes were cultured on tissue culture plastic coated with different extra-cellular matrix proteins. The effects upon monocyte apoptosis of laminins, collagen I and IV and fibronectin, were investigated. 15 isoforms of mammalian laminin proteins are documented, composed of heterotrimers of α, β and γ chains, assembled into cross-shaped molecules (Aumailley et al. 2005). Commercially prepared tissue culture plastic coated with ECM proteins was used for monocyte adhesion, with a mixed composition of laminin proteins or fibronectin proteins. Mononuclear cells were allowed to adhere to matrix-component-coated

tissue culture plastic immediately after isolation, with no previous activation or maturation having taken place, and then treated in serum-free conditions with the same concentrations of cyPGs. Collagen I appeared to confer partial protection against apoptosis induced by Δ12PGJ2 at 10μM (2-way ANOVA, $p<0.001$, $n=4$). However, no reduction in apoptosis was noted following adhesion to collagen IV, laminins or fibronectin (Table 3.14, Figure 3.17). Responses to increasing concentrations of cyPG were assessed separately for monocytes adherent to laminin and fibronectin. Monocytes adherent to fibronectin showed significantly increased apoptosis relative to control treatments, and relative to serum-replete culture, but only at cyPG concentrations above 10μM (2-way ANOVA, $p<0.0001$, $n=4$) (Table 3.15, Figure 3.18). For laminin adhesion, significant increases in apoptosis in serum-free culture compared to serum-replete culture were noted (2-way ANOVA, $p<0.0001$, $n=4$). An elevated level of monocyte apoptosis was seen in control conditions on laminin compared with tissue culture plastic (2-way ANOVA, $p<0.01$, $n=4$). (Table 3.16, Figure 3.19).

3.2.14 Presence of PPARγ increases with monocyte maturation

To examine whether monocyte expression of PPARγ was altered by LDL, freshly isolated un-stimulated purified monocytes were cultured in serum-replete conditions 7 days. Whole cell lysates sampled at 24hrs and 7 days were examined for the presence of PPARγ by immunoblotting. PPARγ was not detectable in monocyte lysates taken from control cells, or cells treated with ox-LDL at 24 hours (Figure 3.20). An increase in PPARγ expression was noted in both ox-LDL treated and control monocytes matured to a 7-day macrophage phenotype, a time-point where susceptibility to pro-apoptotic stimuli had diminished (see section 3.2.12). These data suggest PPARγ may have no role in monocyte apoptosis. They also suggest that PPARγ may counter-regulate apoptosis in monocyte/macrophages.

3.2.15 PPARγ mediated responses depend on monocyte maturity

If PPARγ is only expressed in mature monocytes, PPARγ responses should be maturation dependent. Transcriptional up-regulation of CD36 has been reported to be a reproducible marker of PPARγ ligation (Nagy et al. 1998), conferring increased lipid handling capabilities as well as augmented phagocytic abilities upon individual monocytes as they progress towards a macrophage phenotype. Monocytes cultured in suspension for increasing time periods in the presence or absence of ciglitazone at 10μM were assessed for CD36 expression. Monocytes exposed for less than 24 hours showed a low level of antibody binding with or without ciglitazone treatment. Monocytes matured for 5 days in suspension culture followed by 24hrs of ciglitazone exposure demonstrated enhancement of CD36

expression (Figure 3.21). Specific antagonism of PPARγ with GW9662 completely abolished the ciglitazone-mediated enhancement of CD36 in these cells (Figure 3.22). Thus only mature monocytes appear able to functionally respond to PPARγ ligation. The effects of ox-LDL upon monocyte CD36 expression are explored in detail in Chapter 6.

3.2.16 PPARγ antagonists do not alter apoptosis in mature monocytes

The possibility that PPARγ exerted protection from apoptosis might explain why undifferentiated monocytes with low levels of PPARγ readily underwent apoptosis, whereas mature cells with high levels of PPARγ intra-nuclear protein were less susceptible to cell death. To test whether PPARγ conferred protection from apoptosis, monocyte-macrophages matured for 6 days were pre-treated with the PPARγ antagonist GW 9662 at 10μM for 24 hrs to inhibit PPARγ activity. Following this pre-treatment, cells were again exposed to cyPGs at 10μM for 24 hours before morphological assessment of apoptosis. No statistical difference in apoptosis was noted with the GW9662 compound (2-way ANOVA, n=4, p=0.4294) (Table 3.17, Figure 3.23).

3.2.17 Cyclopentenones do not alter calcium flux in naïve monocytes

Transient calcium influx may be responsible for the alterations in mitochondrial membrane potential that are a causative trigger in programmed cell death (Cerella et al. 2003). Other inflammatory mediators such as leukotriene B4 (LTB4) cause a rapid release of membrane-bound calcium at physiologically relevant concentrations. The pool of calcium affected by LTB4 is the same as that released by other chemotactic factors such as formyl-methionyl-leucyl-phenylalanine (f-Met-Leu-Phe/fMLP) (Naccache et al. 1984). The effect upon calcium potentials across monocyte plasma membranes was assayed after monocyte exposure to $15dPGJ_2$ at 10μM. Freshly isolated un-stimulated monocytes were assayed using a Perkin-Elmer spectrophotometer. Positive control stimuli including fMLP and LTB-4 induced calcium transient fluxes across monocyte cell membranes. However, immediate prior treatment of cell with $15dPGJ_2$ failed to alter calcium potentials, suggesting that cyPG-induced monocyte apoptosis is not mediated by cation flux. Demonstration that the system was functional was provided by the positive inward calcium flux seen after cell permeabilisation with digitonin and the ready reversal of this phenomenon after treatment of the same monocyte samples with the cation-chelating agent EGTA (Figure 3.24, control data not shown). These data are presented as qualitative data representative of experiments performed on two separate monocyte samples.

3.2.18 Cyclopentenones block I-κB degradation in naïve monocytes

Cellular transcriptional activation *via* NF-κB acts to block default apoptosis in leukocytes, an observation made in human peripheral blood polymorphonuclear granulocytes (Ward et al. 1999) (see section 3.1.8). To ascertain whether this inhibition of NF-κB activation accounted for cyPG mediated apoptosis, monocytes were pre-incubated in cyPGs for 1 hour and then exposed to TNF-α or LPS. Lysates prepared from untreated cells demonstrated clear I-κB bands by immuno-blotting (Figure 3.25). Pre-treatment of monocytes with cyPGs $\Delta 12PGJ_2$ and $15dPGJ_2$ at 10μM for one hour increased the strength of immuno-blotting signal seen for I-κB. TNF-α and LPS treated positive controls degraded I-κB after 15 minutes and 30 minutes incubation respectively. TNF-α mediated I-κB degradation was partially reduced by cyPG treatment, whereas LPS induced I-κB degradation was unaffected, raising the possibility that cyPGs may act at a novel checkpoint of the NF-κB activation pathway in monocytes, upstream of I-κB.

3.2.19 Summary of findings

Monocytes undergo LDL mediated apoptosis dependent upon LDL oxidation status.

Monocyte apoptotic responses to LDL are concentration dependent, and dependent on serum withdrawal.

Ox-LDL induced monocyte apoptosis appears to be partially caspase dependent.

Ox-LDL induced monocyte apoptosis is not directly controlled by PPARγ.

Monocyte apoptosis is induced by the cyPGs $\Delta 12PGJ_2$ and $15dPGJ_2$, in a serum-dependent and concentration dependent manner.

cyPG induced apoptosis is not PPARγ dependent, but is mediated by an inhibition of NF-κB activation.

Monocyte maturation confers resistance to cyPG mediated apoptosis in aged macrophages.

Maturation dictates the expression and functional response of intracellular PPARγ in monocytes.

3.3 Technical considerations

3.3.1 Monocyte isolation techniques

Monocytes were isolated from whole blood by density gradient centrifugation, followed by immuno-magnetic separation. This additional step increased purity at the expense of adding complexity to the isolation protocol. However, lymphocyte-derived cytokines may influence monocyte activation and survival (Lopez et al. 1993). Vitronectin receptor-mediated engagement and subsequent phagocytosis of apoptotic cells promotes monocyte survival, the result of simultaneous activation of Akt and inhibition of the mitogen-activated protein kinases extracellular signal-regulated kinase (ERK)1 and ERK2 (ERK1/2) (Reddy et al. 2002). Contaminating granulocyte and lymphocyte may undergo constitutive early apoptosis, and be ingested by monocytes. Cell purity was thus a critical part of isolating an un-stimulated monocyte population for subsequent analyses of monocyte apoptosis induction.

Immuno-magnetic separation is now an established means of producing purified populations of leukocytes by a negative selection process, avoiding cell loss issues that occur in counter-current elutriation (Lund et al. 2000). Immuno-magnetic separation has been reported to produce lower levels of lymphocyte contamination and maintain stable monocyte adhesion molecule expression, in comparison to adhesion mediated purification of monocytes (Nohe et al. 2002). This makes immuno-magnetic separation an attractive option for isolating monocytes, with monocyte purities of 90-95% published in studies on monocyte-derived DCs (Hafsi et al. 2004; Hu et al. 2004). For these reasons, this method of purification was used in preference to elutriation or adhesion-based techniques.

3.3.2 Methods of apoptosis assessment

Markers of apoptosis include terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling, (TUNEL) to demonstrate DNA damage, and assays of caspase activity (Tzima et al. 2005). However, certain modes of apoptosis may be caspase independent and TUNEL labelling is often non-specific, necessitating other modalities for quantification (Grasl-Kraupp et al. 1995). DNA damage may be demonstrated by electrophoresis, an established tool that has shown LDL mediated apoptosis in monocytes (Hutter et al. 2004). However, this would involve the use of nucleosomal ELISA assays to provide an indirect means of quantification (Salgame et al. 1997). Moreover, DNA fragmentation is not exclusive to apoptosis, being present in necrotic cells (Collins et al. 1992). Phosphatidylserine (PS) exposure correlates with apoptotic cell death, reflecting loss of asymmetric PS distribution at the plasma membrane. However, PS exposure may not be truly specific in representing apoptosis. Cells undergoing oncosis, the early stage of cell swelling during primary necrosis,

have been shown to display PS by flow cytometry (Lecoeur, Prevost, & Gougeon 2001). Simpler methodologies, including counting cell nuclei bearing the hallmark condensation morphology seen in apoptosis, are well established. Evidence of nuclear damage during apoptosis is often best visualised by microscopy, with data from electron, fluorescent and light microscopy being robust and consistently corroborated (Kerr, Wyllie, & Currie 1972). Direct visualisation of cells allows the ability to count cells that have passed into post-apoptotic stages of fragility and necrosis (Majno & Joris 1995) that might be missed during processing for flow cytometry to assess Annexin V binding. This type of swollen cell appearance with extrusion of nuclear contents may be a function consequence of failed phagocytic clearance of apoptotic cells, resulting in the production of post-apoptotic ("ghost") cell bodies (Fink & Cookson 2005). Preparation of cells for flow cytometry by washing may alter total numbers of cells seen, causing removal of fragile "ghost" cells and late-apoptotic cells, and thus underestimate the levels of apoptosis seen. It should also be noted that the percentage of cells labelled positively with Annexin V in Figure 3.3 is higher than the percentage apoptosis assessed by microscopy. This may suggest that Annexin V is more sensitive than microscopy in terms of apoptosis detection. However, variable binding of Annexin V may be induced by buffer composition, and there is also a possibility of apoptosis induction during cell sampling and preparation for flow cytometry. Variable survival was also noted in early counts of recovered cells from suspension culture (section 3.2.1). For these reasons, although Annexin-V binding on suspension cells would have been a more technically expedient means of assessing apoptosis, it was decided to measure apoptosis in adherent cells fixed immediately after treatments without washing. These cells were labelled with fluorescent dyes that intercalate with nucleic acids, highlighting condensation of nuclear material. This was the criterion for judging apoptosis. Data for these experiments were then collected by manual counting using fluorescent microscopy, offering the highest level of accuracy for apoptosis assessment.

3.3.3 Activity of reagents used

3.3.3.1 Oxidation status of LDL

LDL was initially isolated locally from individual donors. However, routine limulus amoebocyte lysates testing revealed levels of LPS that were sufficient to cause monocyte activation (data not shown). This necessitated the use of commercial LDL with LPS contamination less than 8 femtogram/ml, (both n-LDL and ox-LDL), to minimise alterations in monocyte viability secondary to activation. Oxidation status was confirmed by gel electrophoresis (Chapter 3 appendix), a more reliable method than assessment of

thiobarbituric acid reactive species (TBARS) (Moore & Roberts 1998). More reliable alternative assays such as gas chromatography, mass spectrometry or immunoassays for isoprostane lipid peroxidation products were not available. Commercially sourced ox-LDL showed marked electrophoretic mobility in comparison to n-LDL but also to locally isolated and oxidised LDL. Commercial LDL is pooled from multiple donors. This avoids issues of individual variation in apoprotein fatty acid chain composition that might affect monocyte responses (Kim et al. 2005). Commercial LDL also avoided issues of matching LDL samples to individual donors, which would have severely limited progress with experiments. The use of copper oxidation of LDL allows production of markedly oxidised LDL species, in an attempt to reflect divalent metal-ion oxidation *in vivo* (Heinecke, Rosen, & Chait 1984), and copper-oxidised LDL has been used to examine monocyte responses in other studies (Burkitt 2001). However, the use of minimally modified LDL and enzymatically-oxidised LDL would have offered a wider view of the actions of modified LDL effects upon monocytes, perhaps more accurately reflecting the actions of LDL degradation in the vessel wall.

3.3.3.2 Activity of PPARγ ligands: thiazolidinediones, GW9662 antagonist and cyclopentenones

PPARγ activation by thiazolidinedione ligation enhances CD36 expression, a phenomenon reported in monocyte/macrophages (Tontonoz et al. 1998) and adipocytes (Yu et al. 2003). Ciglitazone reproducibly induced CD36 expression in cultured monocytes, indicating functional activity of this agent. The GW9662 antagonist produced specific inhibition of CD36 up-regulation, and has been reported to inhibit PPARγ activity in ECs at lower concentrations than those described in this chapter (Polikandriotis et al. 2005). GW9662 has been subsequently shown to be highly specific for PPARγ alone, removing concern that this agent might be acting through other PPAR receptors or that it might be acting as a partial agonist (Seimandi et al. 2005). Studies in ECs utilised luciferase reporter sequences to verify agonist or antagonist effects upon PPARγ. This relies on the use of cell lines as targets for experiments and was thus not relevant to work on primary cells.

3.3.3.3 Activity of caspase inhibitor

The pan-caspase inhibitor z-VAD-fmk non-specifically inhibits caspases. Thus the existence of caspase dependence in apoptotic processes under study may be discerned but no further distinction is possible. z-VAD-fmk compound has a t½ of 40mins at a concentration of 1μM (Garcia-Calvo et al. 1998), and it is unlikely that activity was lost during the duration of these experiments, which used 100μM concentrations. Care was taken to ensure that

preparation of z-VAD-fmk occurred immediately prior to each assay, from stocks used successfully in the inhibition of apoptosis in granulocytes (Ward et al. 2002).

The dependence of monocyte apoptosis upon caspases has been explored in detail (Sun et al. 1999) and is discussed further in section 3.4.

3.3.4 Study limitations

3.3.4.1 Time course of apoptosis

Apoptosis was assessed after at least 24hrs in adherent culture. By using shorter time intervals of 2-4hrs, the induction of apoptosis could have been followed more closely. This would help gauge appropriate time-points for further experiments, especially biochemical investigations of apoptotic mechanisms, and alterations in cell phenotype during apoptosis.

3.3.4.2 Adhesion to matrix proteins

Adhesion of monocytes to matrix proteins was followed by immediate exposure to cyPGs. This may not have allowed sufficient time to elapse to permit integrin ligation, and thus differential adhesion-mediated effects upon apoptosis may have been missed. It might be useful to explore the effects of pre-adhesion to specific matrices by examining time-courses of adhesion, prior to the addition of pro-apoptotic reagents. This might be done in a parallel fashion to the maturation studies on tissue culture plastic, and would highlight whether a critical duration of matrix adhesion was necessary to offer protection from apoptosis.

3.3.4.3 Assessment of NF-κB

Assessment of NF-κB activation was inferred from immuno-blots of I-κB, on the assumption that I-κB degradation intrinsically reflects release of NF-κB subunits. Although this is a validated means of assessing NF-κB activation, a more definitive method of clarifying this would be to use electromobility shift assays to assess nuclear translocation of the p65 subunit. Further insight into this system might be offered by enhancing cytoplasmic levels of I-κB (Fujihara et al. 2005) or by altering phosphorylase activity that regulates I-κB degradation (Karin & Ben Neriah 2000).

3.3.4.4 Examination of serum effects

The induction of apoptosis dependent upon serum withdrawal did not address issues of individual serum components conferring a survival benefit. Alteration of serum composition or exploration of individual candidate cytokines including in serum-free culture would

enable a more comprehensive assessment of the factors responsible for protection against apoptosis.

The issue of LDL in serum from donors was also considered. LDL concentrations were not available, due to constraints on clinical data collection in the ethics approval. However, autologous serum was added at 10%, limiting the levels of LDL in culture media. Furthermore, distinct effects following ox-LDL supplementation were visible. De-lipidated non-autologous serum was deliberately not used, to minimise monocyte activation.

3.3.5 Further experiments

The studies presented provide a foundation for more detailed mechanistic analysis of ox-LDL-induced apoptosis. Closer examination of apoptosis induction in monocytes at earlier time points could be undertaken, with apoptosis counts performed at 4hr intervals initially. A time course of maturation needs to be performed with ox-LDL treated samples, to parallel work on maturation performed with cyPGs.

The mechanism underlying adhesion to specific matrices followed by LDL exposure needs to be assessed. The use of different ox-LDL species should be considered, with alternative sources of ox-LDL and minimally modified LDL used. In particular, the use of enzymatically altered LDL e.g. through the actions of 12/15 lipoxygenase, would offer an insight into potential *in vivo* LDL species, and would be more physiologically relevant than copper oxidised LDL alone.

The biochemistry of both cyPG and ox-LDL mediated monocyte apoptosis demands further exploration. Inhibition of individual caspases and an assessment of individual caspases' activity are necessary. An attempt was made to assess caspase 3 activity in monocyte lysates by immuno-blotting, but was unsuccessful. This merits repetition, in particular to assess the apparent caspase independence of ox-LDL-mediated apoptosis. Further information as to the apoptotic paths followed by LDL exposed monocytes would be gained from assessment of changes in mitochondrial membrane potential, using fluorochrome-linked markers specific for apoptosis such as JC-1 (Mancini et al. 1997).

The role that regulatory enzymes controlling NF-κB-mediated monocyte activation play in modulating monocyte apoptosis requires further study. The mechanism by which cyPGs alter phosphorylation of I-κB requires exploration of the activity of I-κB kinase (IKK) by immuno-blotting, and by curcumin-mediated inhibition of IKK (Plummer 1999). Ox-LDL appeared to show gross degradation of I-κB by immuno-blotting (data not shown), suggesting that apoptotic effects may occur despite NF-κB activation. This observation would benefit from repetition. As for cyPG mediated apoptosis, the specific blockade of

individual components of NF-κB activation would illustrate whether ox-LDL mediate apoptosis is NF-κB independent.

The role of PPARγ in monocyte survival has been examined using a specific antagonist. Further assessment of PPARγ activity in the context of monocyte apoptosis could be made using a number of gene silencing strategies. These are discussed in detail in Chapter 4.

3.4 DISCUSSION

3.4.1 LDL induction of monocyte apoptosis

Several previous studies have been made of monocyte survival following exposure to lipid-derived compounds. Initial reports showed that U937 and HL60 monocytic cell lines undergo apoptosis induced by 7 beta-hydroxycholesterol (Aupeix et al. 1995). Further evidence showed that human primary monocyte exposure to ox-LDL resulted in ultra-structural changes consistent with apoptosis (Hardwick et al. 1996). Studies have focused on the susceptibility of free-cholesterol loaded macrophages to succumb to apoptosis (Yao & Tabas 2000). However, this is in distinction to other data that suggest a direct influence of ox-LDL upon monocyte survival influence monocyte death *via* a CD36-mediated binding of ox-LDL leading to a caspase-3 mediated death pathway (Wintergerst et al. 2000). This is in keeping with the data presented in this chapter showing LDL differentially induced monocyte apoptosis dependent upon oxidation status. The lower level of observed apoptosis with native LDL (section 3.2.4), suggests that receptor specificity may play a part in survival responses. Validation of the uptake of different lipid species is difficult to perform and to quantify, other than by radio-labelling strategies. The possibility of individual receptor ligation being responsible for apoptosis induction is of interest. Studies using anti-CD36 blocking antibodies would be useful in this regard, but were not performed due to time constraints. A more definitive experiment would be to use CD36-null murine monocytes (Febbraio et al. 1999). Human CD36 null subjects are documented, but cells from such subjects are extremely difficult to access, and this option was not practical.

Further contributory factors to plaque degradation and vulnerability relate to the thrombogenic profile that areas of monocyte apoptosis may represent, including the production of tissue factor as a direct result of ox-LDL induced caspase-mediated monocyte death (Hutter et al. 2004). Further evidence that ox-LDL mediated death is relevant to atherosclerotic progression has been gained from observations on aortic lesions in LDL R-/- mice. Deletion of the survival protein apoptosis inhibitor expressed by macrophages (AIM) in LDL R-/- animals resulted in enhanced macrophage susceptibility to ox-LDL induced

apoptosis, reducing aortic plaque volume (Arai et al. 2005). This latter observation is also of interest as AIM is under the transcriptional regulation of LXR/RXR heterodimers, nuclear transcriptional regulators with similar functions to PPARγ.

3.4.1.1 Oxidation and monocyte apoptosis

The role of ox-LDL in the general process of atherosclerosis has been debated. Observations from clinical studies suggest that systemic anti-oxidant therapies show no benefit in vascular disease, calling into question the role of ox-LDL not only in the context of monocyte survival, but also in atherosclerosis *per se*. The issues here are complex, and may relate to non-specific blockade of oxidation pathways governing LDL modification, and inadequate inhibition of oxidative enzymes at a micro-environmental level. It is more likely that pro-atherosclerotic oxidative stress occurs in localised inflammatory sites at the vessel wall, rather than being a systemic process (reviewed in Stocker & Keaney, Jr. 2004). These data are made still more difficult to interpret in the face of evidence that low concentrations of ox-LDL are anti-apoptotic in macrophages, where survival is promoted by ox-LDL mediated activation of the phosphokinase Akt (Hundal et al. 2001). The absence of caspase dependent apoptosis does suggest that oxidation state important in ox-LDL mediated death. Evidence from ECs shows that superoxide dismutase limits ox-LDL induction of apoptosis, suggesting that surface membrane oxidative stress may be a trigger to apoptosis (Harada-Shiba et al. 1998).

3.4.2 PPARγ role in apoptosis

The role of PPARγ in ox-LDL mediated effects upon monocytes is complex. Functional changes have been demonstrated using ox-LDL itself as well as metabolites that have been identified as natural ligands for PPARγ ((Nagy et al. 1998) see also Chapter 1, section 1.20). The concept that ox-LDL was mediating apoptosis *via* PPARγ seemed less intuitive. If this were true, it would cause monocyte deletion, precluding the invocation of pro-atherosclerotic responses that produce plaque progression. Furthermore, the lack of inhibition of apoptosis using the GW9662 PPARγ antagonist suggested a differential mechanism. Further evidence that ox-LDL monocyte apoptosis was PPARγ-independent was provided by studies using 13(S)HODE, demonstrating lower levels of apoptosis in comparison to ox-LDL mediated effects. 13(S)HODE may induce apoptosis in colonic carcinoma cell lines, and the limited monocyte apoptosis seen in section 3.2.7 may be representative of a similar mechanism.

The role of PPARγ in cyPG-mediated apoptosis posed similar problems. This class of ligand is pharmacologically active *in vitro* at 10μM, concentrations that are reported as

physiological and that are sufficient to initiate PPARγ-mediated signalling (Straus & Glass 2001). However, there is controversy in the literature regarding the relevance of cyPGs *in vivo*, with some studies suggesting that pharmacologically active doses of $15dPGJ_2$ are not seen in biological or pathological systems, with particular regard to inflammatory sites (Bell-Parikh et al. 2003). Without the ability to sample at a cellular level, it is difficult to refute the suggestion that high concentrations of these cyPGs might be present in vascular wall lesions, representing a gradient of inflammatory mediators at sites of active injury. Other reports confirm cyPG mediated apoptosis in inflammatory cells, albeit at higher concentrations than used in the experiments presented in this chapter, and suggest that thiazolidinediones also mediate apoptosis, *via* PPARγ (Kawahito et al. 2000). cyPG-mediated apoptosis has been described in B cells (Piva et al. 2005) and granulocytes (Ward et al. 2002) with the common finding that cyPGs blocked cell survival *via* PPARγ-independent mechanisms, specifically limiting NF-κB translocation. Certainly the lack of apoptotic induction with thiazolidinediones in monocytes (section 3.2.8) fits with this, as does the failure of the GW9662 PPARγ antagonist to protect monocytes from cyPG mediated apoptosis (section 3.2.6). Immuno-blotting data showing negligible levels of intra-cellular PPARγ in naïve monocytes capable of undergoing cyPG-mediated apoptosis (section 3.2.14), and the absence of a functional PPARγ-mediated response in monocytes at a similar stage of culture (section 3.2.15) also strengthen the argument that PPARγ is not an apoptotic mediator in these cells.

3.4.3 Protection from apoptosis

3.4.3.1 Serum influences upon apoptosis

The serum dependence of monocyte survival has been documented *in vitro* (Mangan & Wahl 1991). Putative mechanisms that mediate this may involve heat-shock protein 70 up-regulation (Lang et al. 2000). Detailed examination of individual serum components conferring monocyte survival was not embarked upon. However it is likely that certain key molecules are important in this regard. GM-CSF has been shown to up-regulate anti-apoptotic protein expression in granulocytes (Kobayashi et al. 2005), and similar effects upon ox-LDL mediated monocyte apoptosis would be of interest. Differential T lymphokine influences also dictate monocyte survival with IL-12 protecting against apoptosis in contrast to IL-10 promoting apoptosis (Estaquier & Ameisen 1997). This would be of particular interest in ox-LDL treated cells, in the context of potential signals derived from T cells within atherosclerotic plaque. Pro-survival signals from serum include the nuclear translocation of NF-κB (section 3.1.8) in association with p38 mitogen-activated protein

kinase (MAPK) (Park et al. 2002). NF-κB promotes survival by enhancing levels of anti-apoptotic bcl proteins in macrophages (Liu et al. 2004). Differential expression of bcl-2 governs the individual susceptibility of lymphocytes monocytes and granulocytes to Fas-mediated apoptosis (Iwai et al. 1994), and expression of bcl proteins may change during monocyte maturation.

3.4.3.2 Maturation governs monocyte apoptosis

Ingestion of free cholesterol has been associated with the induction of macrophage apoptosis, (Yao & Tabas 2001). The effects of ox-LDL upon monocyte apoptosis were tested on early adherent monocytes (sections 3.2.2-3.2.4). However, subsequent experiments presented here suggest that mature macrophages are less susceptible to apoptosis, albeit that induced by cyPGs (section 3.2.12). Maturation dependent alteration of monocyte apoptosis is consistent with data published by other groups, demonstrating critical changes in pro-and anti-apoptotic protein balance (Perera & Waldmann 1998). Mature cells may display augmented surface expression of $\alpha v\beta_3$ (Scatena et al. 1998) and CD16 molecules (Wang et al. 2001), and downstream signalling following ligation of these molecules can confer a survival advantage. The role of the bcl family of proteins has been highlighted in the inhibition of constitutive and induced apoptosis (Milner, Johnson, & Gregory 1992). Evidence that ox-LDL directly mediates bcl-2 suppression in ECs (Li, Yang, & Mehta 1998), and governs VSMC apoptotic responses (Bennett 1999), suggests a role for bcl proteins in the control of cell death within atherosclerotic plaque. Although bcl proteins were not measured in these studies, further assessment of ox-LDL mediated monocyte apoptosis would necessitate quantification of their expression.

3.4.3.3 NF-κB activation in monocyte apoptosis

The NF-κB signalling cascade directs cell activation and survival, (reviewed in section 3.1.8). Cell survival is augmented by NF-κB directed transcription of anti-apoptotic proteins such as survivin (Zhu et al. 2001), and the IAP c-IAP2 (Chu et al. 1997) to limit TNFα-mediated apoptosis. NF-κB nuclear translocation is cyclical, allowing rapid responses to stimuli, facilitating responses to inflammatory injury and microbial infection. I-κB participates in an equally rapid regulatory feedback cycle, to limit NF-κB mediated responses, making I-κB critical to innate immunity and leukocyte survival, (reviewed in Ghosh, May, & Kopp 1998). I-κB phosphorylation is regulated by the I-κB kinase (IKK) complex, comprising IKKα, β and γ enzymes (Ghosh & Karin 2002). IKKβ regulates

thymocyte apoptosis and IKKα has been shown to inhibit B cell apoptosis (Senftleben et al. 2001).

The reduction in monocyte I-κB degradation seen in response to both LPS and TNFα stimuli following cyPG treatment (section 3.2.18) raises the possibility that these agents may act at the level of the IKK complex or higher. PPARγ ligands act to down regulate IKK-α, limiting NF-κB activation and explaining their anti-inflammatory effects (Castrillo et al. 2001). The immuno-blotting evidence presented shows specific blockade of TNF-α mediated NF-κB activation and suggests an additional higher level of control in this system. An alternative checkpoint may be NIK kinase (NF-κB inducing kinase) (Malinin et al. 1997) which normally interacts with the TNF-receptor associated factor (TRAF)-2 to stimulates I-κBα degradation, and this possibility requires further exploration in monocytes. In summary, in the context of other literature in the field, the findings presented here relating to cyPG monocyte apoptosis suggest that it is driven *via* inhibition of NF-κB activation paths.

3.4.3.4 Adhesion regulates monocyte apoptosis

Adhesion may confer cell survival despite the presence of pro-apoptotic stimuli. Integrin-mediated outside-in signalling promotes biochemical paths that inhibit apoptotic programmes, including the release of mitochondrial cytochrome c in a PI3 kinase/Akt dependent manner (Aoudjit & Vuori 2001). The matrix to which cells adhere is a solid network of proteins, secreted by cells themselves, and rendered insoluble by protein cross-linking. Adhesive proteins such as fibronectins, laminins, and vitronectin are associated with this solid matrix phase, the rigidity of which may play a role in strengthening the integrin-cytoskeleton links (Choquet, Felsenfeld, & Sheetz 1997). Collagen IV and laminins, along with heparin sulphate proteoglycans, form part of the vascular basement membrane, and mediate leukocyte adhesion *via* β1 integrins (Nishiuchi et al. 2006). Type IV collagen is a polyvalent ligand, with sequences that can bind α1β1, α2β1, and α3β1 integrins, as well as cell surface proteoglycan receptors, including CD44/chondroitin sulfate proteoglycan (CSPG) (Baronas-Lowell et al. 2004). VSMC production of collagen I is implicated in fibrous cap formation, and is important in the repair mechanisms following vessel wall injury (Bou-Gharios et al. 2004). Immuno-histochemical studies demonstrate high-levels of collagen I expression in recently degraded or ruptured atherosclerotic plaques (Kolodgie et al. 2002).

Fibronectins also serve as ligands for integrin adhesion receptors. Fibronectin type III (FN III) domains possess the Arg-Gly-Asp (RGD) cell attachment site conferring the ability to bind to β3 integrins (Ruoslahti & Pierschbacher 1986). Proteases capable of fibronectin

degradation have been associated with augmented leukocyte apoptosis (Michel 2003). The interaction between leukocytes and ECM proteins is important in directing the survival as well as the function of leukocytes in inflammatory response. Binding to collagen IV and laminin, but not collagen I or fibronectin, protects mesangial cells from apoptosis *via* a β1 integrin-mediated, but arg-gly-asp (RGD)-independent mechanism, (Mooney et al. 1999).

One explanation for this phenomenon would be that loss of cell numbers might result in low levels of cell-cell interactions. This concept of anoikis or "homelessness" is seen in other cell systems, where a default program of apoptosis is reached because a cell "senses" that it is isolated and has no extrinsic feedback (Hamada et al. 1998).

One possible alternative explanation is that matrix binding of naïve undifferentiated cells invokes apoptosis. This is a phenomenon noted in haematopoiesis, with myeloid pre-cursors still resident in the bone marrow undergoing programmed cell death following VLA5 mediated binding events (Terui et al. 1996). Within the marrow, this represents a homeostatic mechanism to regulate leukocyte production. However, it may adversely affect the survival of early circulating but uncommitted leukocytes. The effects of matrix binding upon monocyte survival demands further investigation. Adhesion to matrices for increasing time periods would allow assessment of prolonged binding upon cell survival. The anti-apoptotic effects of maturation (section 3.4.3.2) might be expected to occur earlier with matrix adhesion, and the data presented do not refute this possibility. Inhibition of individual monocyte surface adhesion molecules would add further insight as to the involvement of adhesion events upon monocyte apoptosis. Finally, an exploration of apoptotic proteins, as suggested in section 3.4.3.2, would be of equal interest following defined adhesion events, in the setting of ox-LDL mediated or cyPG mediated apoptosis.

3.4.4 Relevance of apoptosis to atherosclerosis

Apoptosis is important in all inflammatory processes including atherosclerosis, potentially contributing to plaque core formation, plaque volume and stability (see Chapter 1). Cell responses to apoptosis vary dramatically, with alterations in susceptibility to cell death governed by activation state, maturity and the context within which pro- or anti-apoptotic signals are received. Certain cells may survive given the appropriate stimuli, while others are deleted. It is likely that apoptosis varies in atherosclerotic lesions, with direct relevance to atherosclerotic plaque progression, and this merits discussion.

3.4.4.1 Differential apoptosis dependent on stages of atherosclerosis

Atherosclerotic plaque may display differential levels of monocyte apoptosis dependent upon the stage of atheromatous progression. Monocyte survival within a fatty streak is likely

to be different to an advanced lesion, possibly because susceptibility to apoptosis may alter as plaque complexity increases (reviewed by Geng & Libby 2002). In early plaque, apoptosis may be an appropriate response to pro-atherogenic stimuli. Murine models offer insight into the homeostatic responses to monocyte lipid loading that appear to operate in early atheromatous lesions, with apoptosis modulating lesion cellularity and size. The pro-apoptotic bcl-2 protein Bax appears to be involved in atherosclerotic plaque regulation, with adoptive transfer of Bax-/- myeloid cells into LDL-R-/- mice reducing atherosclerotic plaque volume (Liu et al. 2005). Bone marrow reconstitution of apoE*3-Leiden mice with myeloid cells from p53-deficient mice reduces macrophage apoptosis in early aortic atherosclerotic plaques. Associated increases in plaque volume suggest there is an absolute requirement for macrophages to limit early atheromatous progression *in vivo* (van Vlijmen et al. 2001). The differential susceptibility to apoptosis displayed by monocytes in response to cyPGs suggests that maturation status is a further variable that may govern lesion cellularity and local inflammatory balance. Similar changes may apply to macrophages exposed to differentially modified LDL, suggesting that apoptotic programmes vary according to lesion type, and cell population. Although such data may be considered with regard to cell turnover alone, a key further consideration with regard to apoptotic balance is the role of phagocytic clearance.

3.4.4.2 Monocyte apoptosis and clearance: implications for atherosclerosis

The murine studies discussed suggest that phagocytic clearance mechanisms are still efficient an early stage of atherosclerosis. However, monocyte cell death is a feature of atherosclerotic plaque that is visible in *ex vivo* samples of arteries with advanced lesions (Kockx 1998). This type of plaque also is rich in apoptotic monocytes that surround the necrotic plaque core (Hegyi et al. 1996). A suggestion that impaired clearance of apoptotic cells may be to account for this has been based on observations relating absolute cell counts to failed clearance of apoptotic cells (Schrijvers et al. 2005). Other *in vivo* studies have demonstrated defective phagocytic clearance exacerbates chronic inflammation in disease states with parallels to atherosclerosis, such as systemic lupus erythematosus (Taylor et al. 2000). *In vitro* studies have shown that macrophages laden with ox-LDL display impaired phagocytic capacity for both apoptotic vascular smooth muscle cells (VSMC) and fibroblasts, although phagocytic recognition remains intact. Moreover, ox-LDL loaded macrophages demonstrate enhanced secretion of both MCP-1 and IL-6, promoting local inflammation in the vessel wall (Khan et al. 2003).

A mechanistic link between atherosclerosis and phagocytic clearance may be the shared mechanism for apoptotic cell recognition and ox-LDL binding by scavenger receptors at the

monocyte surface (Sambrano & Steinberg 1995). Antibodies to ox-LDL directly interfere with macrophage binding of apoptotic cells, (Chang et al. 1999), with the implication that phagocytosis may be impaired by specifically modified LDL species. Antibodies to modified LDL that localise to human atheroma have been shown to impair phagocytosis in macrophages (Shaw et al. 2001). Furthermore, minimally-modified LDL limits phagocytosis at a more fundamental level by promoting actin polymerisation and macrophage spreading, *via* a CD14-binding event (Miller et al. 2003).

The consequences of un-cleared apoptosis are relevant to atherothrombosis. Ox-LDL induced monocyte apoptosis is associated with enhanced tissue factor (TF) production (Hutter et al. 2004). Shed membrane micro-particles derived from apoptotic cells have been noted to be pro-coagulant in human atherosclerotic plaque (Mallat & Tedgui 1999). Necrotic core formation from local monocyte/macrophage apoptosis results in lysophosphatidylic acid accumulation, a further stimulus to platelet aggregation (Siess et al. 1999).

Apoptosis is as important in atherosclerosis as in other inflammatory conditions. The influences of inflammatory and atherogenic stimuli may alter lesional cell populations, as well as affecting inflammation and propagation of vascular injury. Further exploration of clearance mechanisms as well as apoptosis induction is needed.

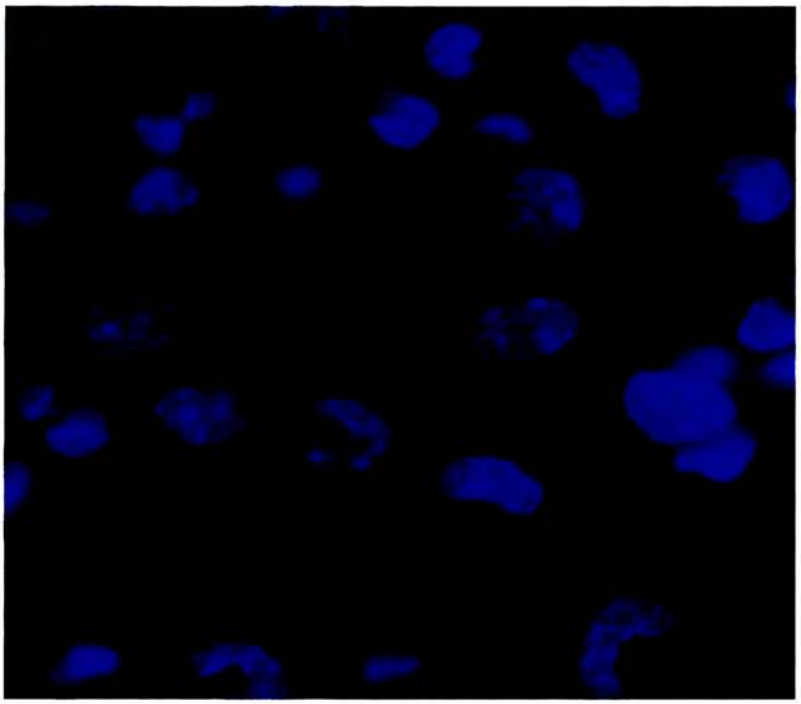


Figure 3-1 Fluorescent micrograph, control monocytes 24hrs

Control adherent monocytes were cultured in serum-free conditions for 24 hours adherent to tissue culture plastic. Cells were fixed with 1% formaldehyde, permeabilised with 1% Triton and nuclear material labelled with 3μg/ml of Hoechst 33428. A characteristic normal open granular nuclear matrix appearance is evident, with a typical "horseshoe" morphology seen in these control cells (x32 magnification).

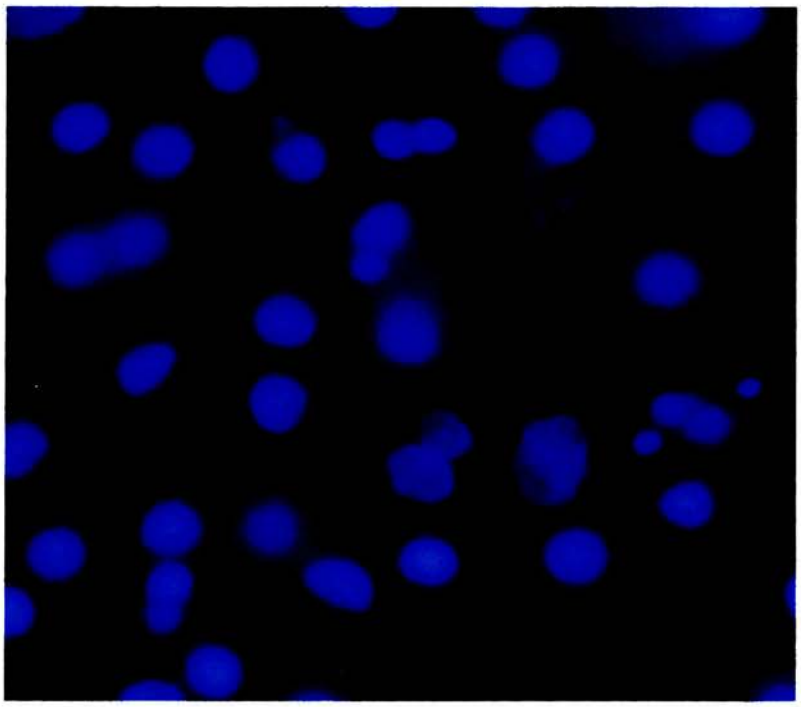


Figure 3-2 Fluorescent micrograph, apoptotic monocytes, 24 hrs

Ox-LDL treated adherent monocytes were cultured for 24 hours in serum-free conditions, adherent to tissue culture plastic. A marked reduction in nuclear size and rounded pattern of nuclear shrinkage is consistent with the DNA damage characteristic of apoptosis (x32 magnification).

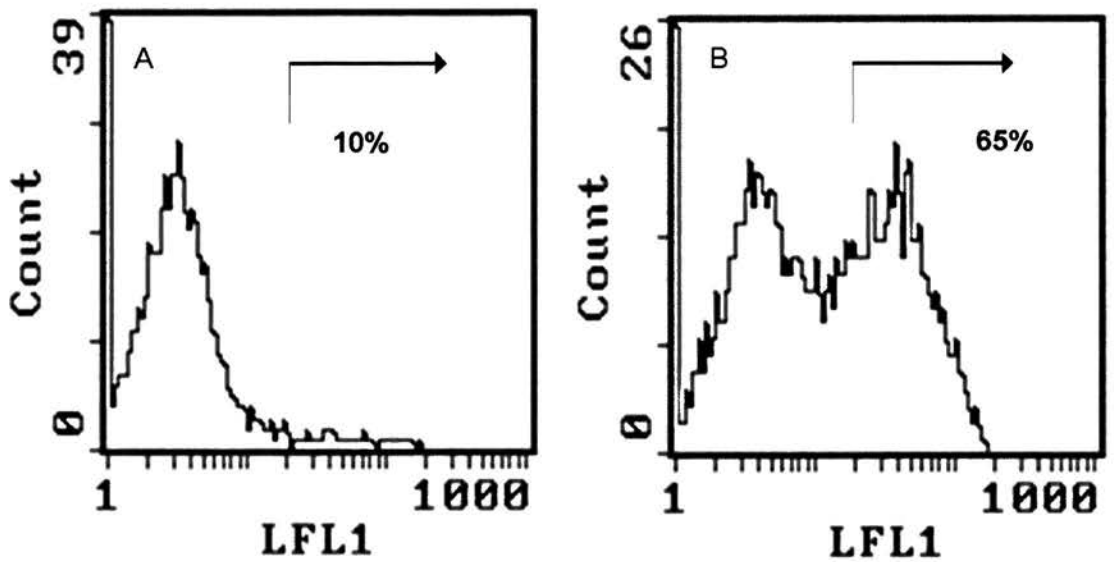


Figure 3-3 Flow cytometric assessment of monocyte apoptosis

Control monocytes were cultured in suspension in serum-free conditions for 24 hours, and subsequently labelled with Annexin V-FITC to demonstrate phosphatidylserine exposure. Control monocytes in serum-free conditions demonstrate a low level of constitutive apoptosis, evidenced by the sub-population comprising 10% of total monocyte numbers that has undergone apoptosis by this measure (Figure 3.3A).

Monocytes cultured in ox-LDL in suspension in serum-free conditions for 24 hours. The same labelling of phosphatidylserine using Annexin V-FITC has been employed. A marked increase in signal is seen, with a clear biphasic signal indicating dual populations of apoptotic and non-apoptotic cells (Figure 3.3B).

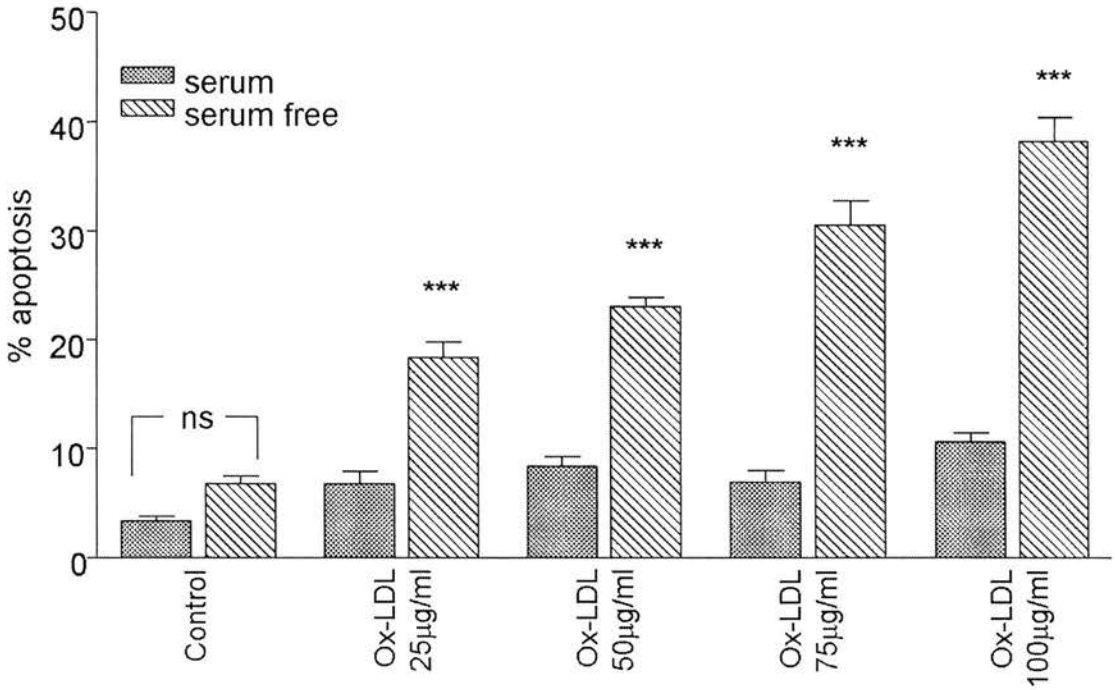


Figure 3-4 Monocyte apoptosis, serum-free culture, 24hrs, ox-LDL supplements

Ox-LDL significantly increases monocyte apoptosis relative to control in a concentration and serum-dependent manner, (2-way ANOVA, $p<0.0001$, $n=4$). Differences in apoptosis between concentrations of ox-LDL, and between serum treated and serum-free monocytes were significant, but not annotated on this figure.

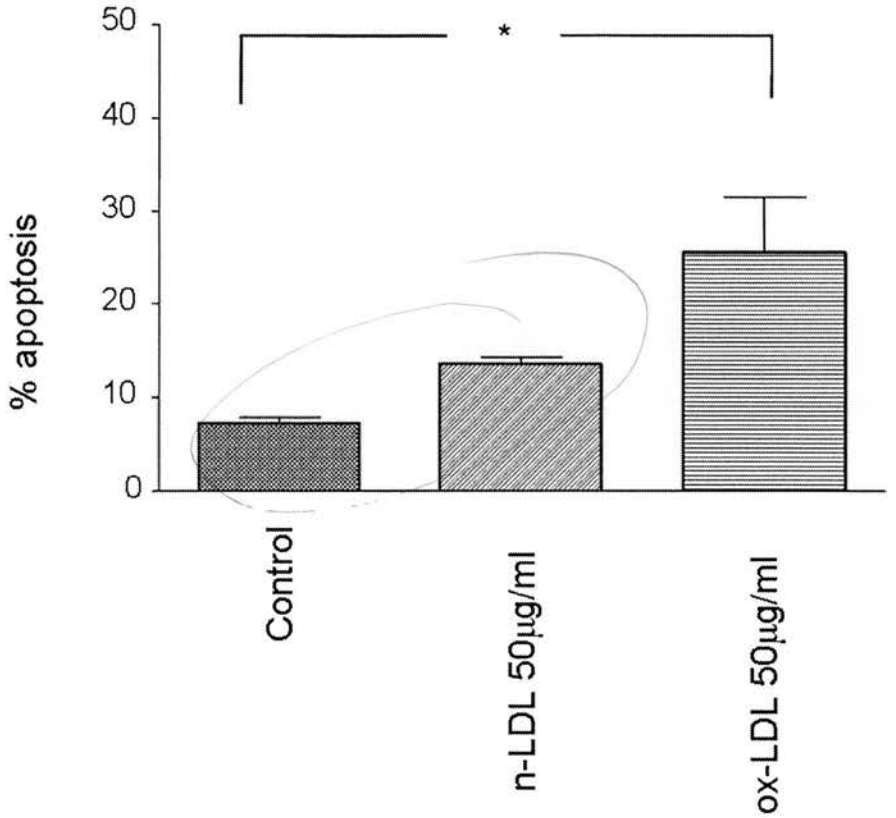


Figure 3-5 Monocyte apoptosis, serum free culture, n-LDL vs. ox-LDL supplements

Monocytes supplemented with 50μg/ml of n-LDL or ox-LDL, were cultured in serum-free adherent conditions for 24hrs. Apoptosis was assessed by nuclear morphology. No significant increase in apoptosis was induced by the presence of n-LDL. Ox-LDL significantly increased apoptosis compared to control, but not compared to n-LDL (Friedman one-way ANOVA, n=4, p=0.0046).

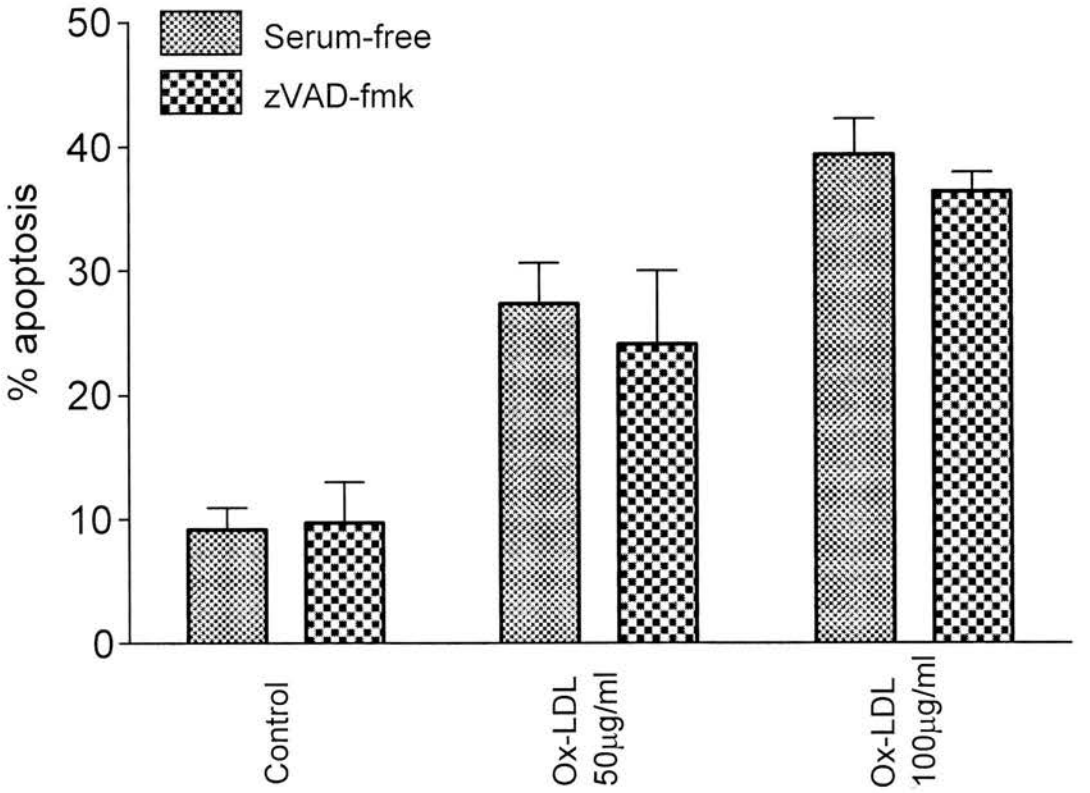


Figure 3-6 Ox-LDL induction of monocyte apoptosis is not fully reversed by the pan-caspase inhibitor zVAD-fmk

Increasing levels of apoptosis were seen during serum-free incubation of monocytes with ox-LDL. Pre-incubation with the caspase-inhibitor benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone zVAD-fmk produced a non-significant reduction in apoptosis (n=4, p=0.7289).

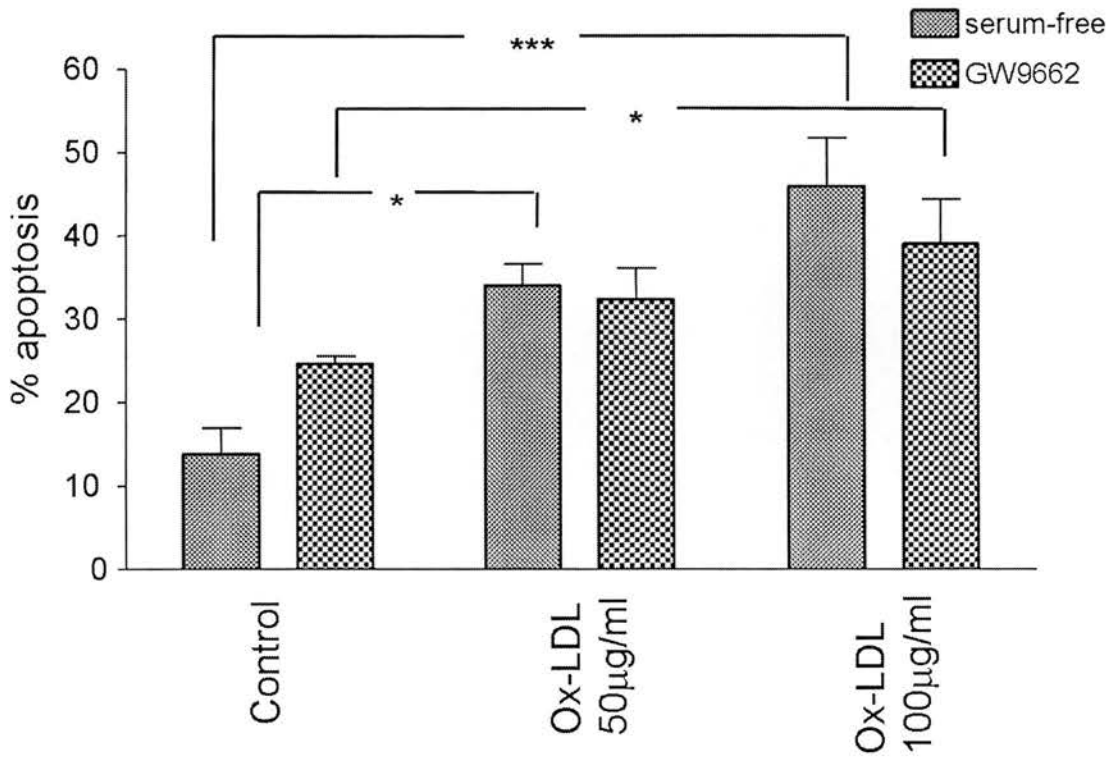


Figure 3-7 Ox-LDL induction of monocyte apoptosis is not reduced by the PPARγ antagonist GW9662

Adherent naïve monocytes were pre-treated with the pharmacological antagonist to PPARγ GW9662. Ox-LDL supplements were added to culture media for 24hrs before assessment of apoptosis, and induced significant levels of apoptosis relative to control. GW9662 treatment produced no significant difference in ox-LDL induction of apoptosis (2-way ANOVA with Bonferroni post-tests, p=0.3532, n=4).

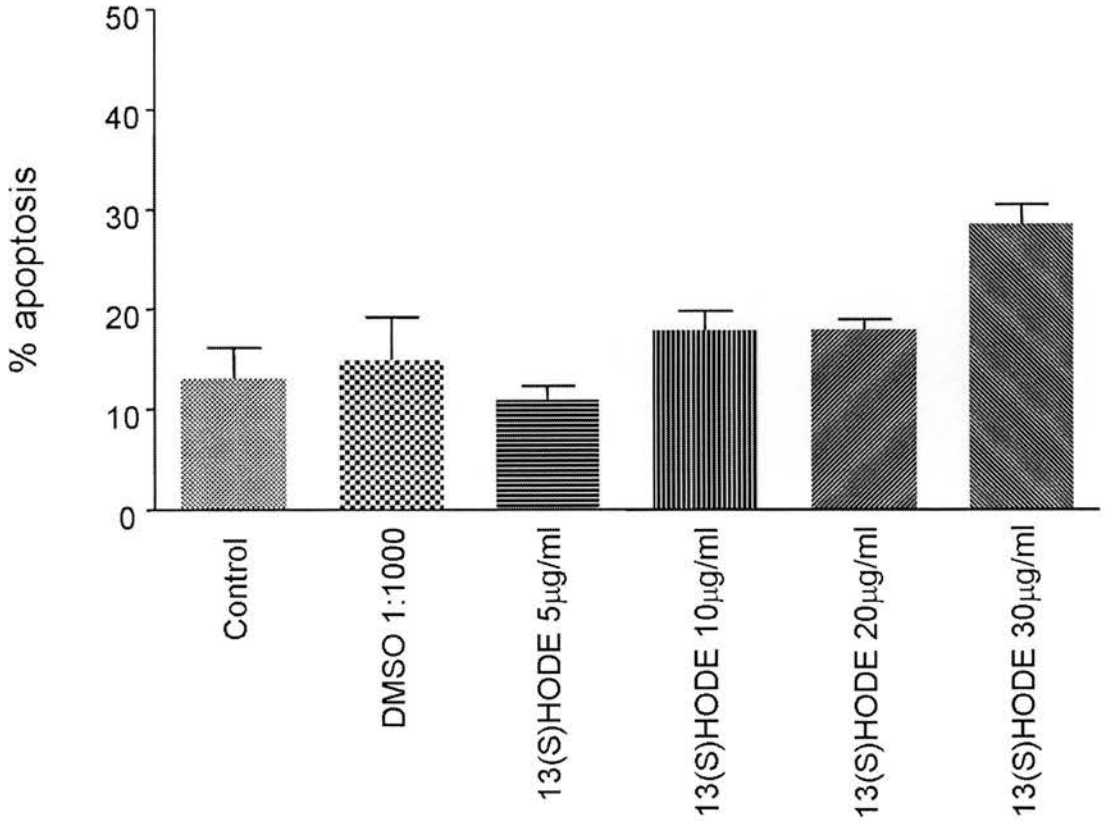


Figure 3-8 Monocyte apoptosis, 24hrs, 13(S)HODE supplements

Adherent monocytes cultured in serum-free conditions were assessed morphologically for apoptosis after 24 hours of exposure to 13 (S) HODE, a product of ox-LDL degradation which has been reported to act as a natural PPARγ ligand. No increase in apoptosis was noted in comparison to control (one-way ANOVA, n=4, p=0.514).

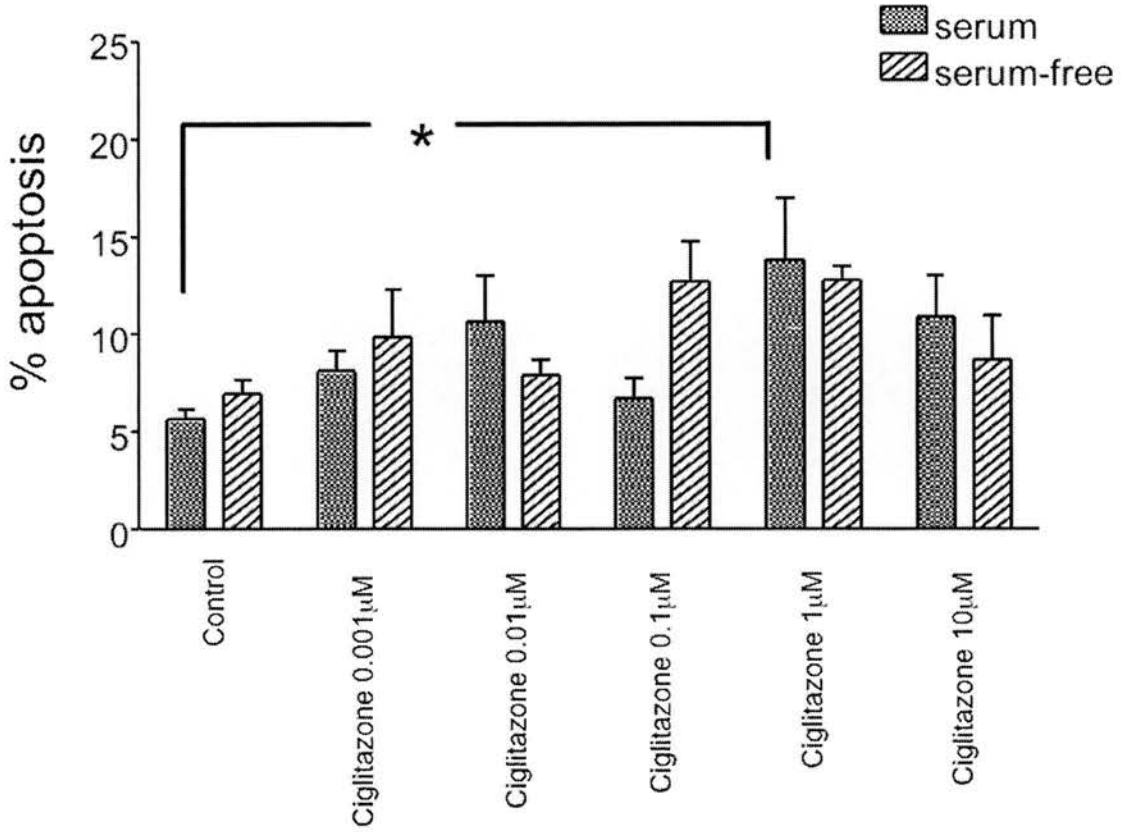


Figure 3-9 Monocyte apoptosis, 24hrs culture, ciglitazone supplements

Increasing concentrations of the thiazolidinedione Ciglitazone caused low-levels of apoptosis. A small significant increment in apoptosis was seen in serum-replete cells with 1μM of ciglitazone, but not with 10μM (2-way ANOVA, n=4, p=0.0217). No significant difference in apoptosis was seen between serum and serum-free groups for each concentration.

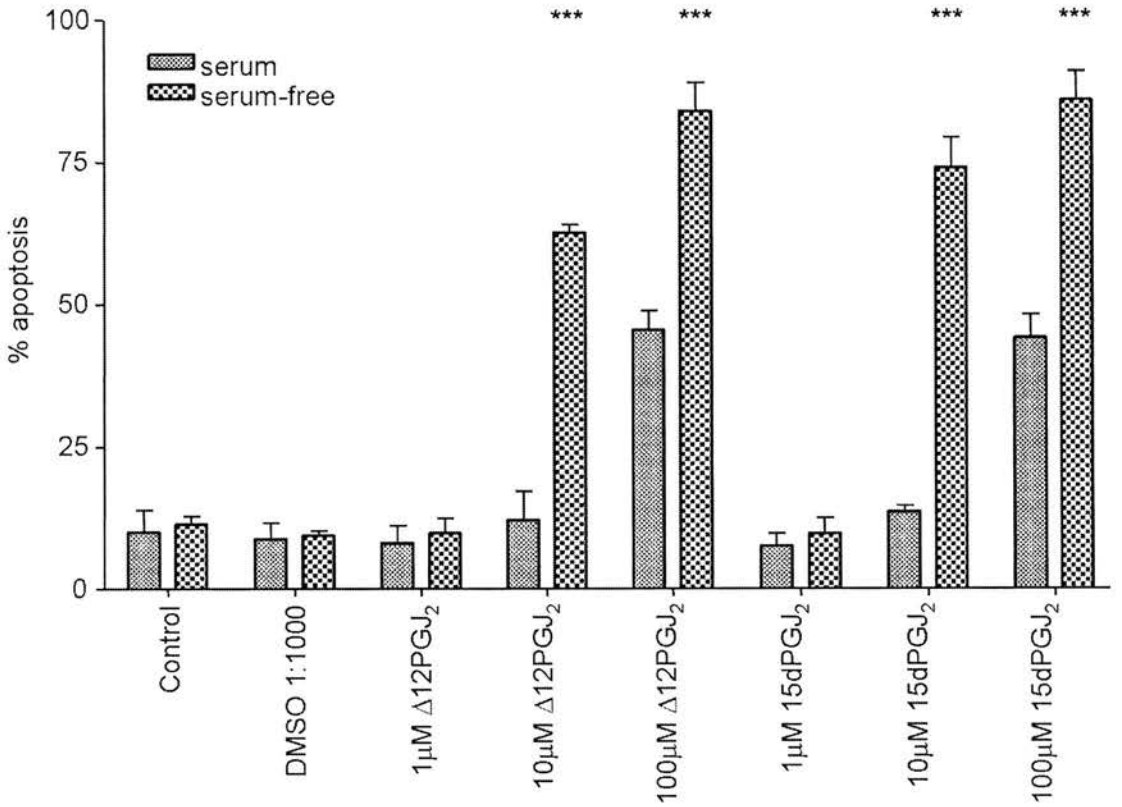


Figure 3-10 Monocyte apoptosis, 24hrs serum-free culture, cyclopentenone prostaglandin concentration responses

Preliminary experiments assessed the concentration of the cyclopentenone prostaglandins Δ12PGJ$_2$ and 15dPGJ$_2$ necessary to induce monocyte apoptosis. Low concentrations produced little elevation in percentage apoptosis over control. The induction of apoptosis by concentrations of both Δ12PGJ$_2$ and 15dPGJ$_2$ was significant (2-way ANOVA, $p<0.0001$, $n=4$) but similar, at a concentration of 10μM and 100μM.

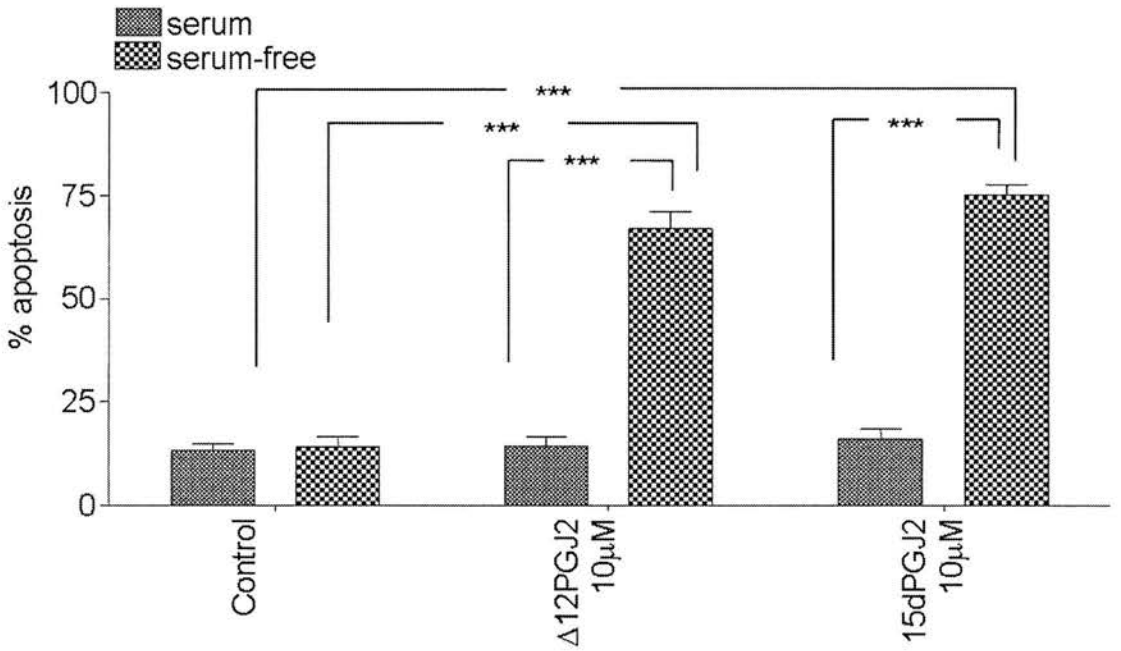


Figure 3-11 Monocyte apoptosis, 24hrs, serum free culture, cyclopentenone prostaglandin supplements

Further data confirmed that cyPGs induced monocyte apoptosis at 10μM, a concentration reported as physiologically active.
Adherent serum-free monocytes were exposed for 24 hours to the cyclopentenone prostaglandins Δ12PGJ$_2$ and 15dPGJ$_2$ at 10μM. Apoptosis was assessed by nuclear morphology. Both Δ12PGJ$_2$ and 15dPGJ$_2$ induced significant levels of apoptosis (2-way ANOVA, Bonferroni post-test, $p<0.0001$, $n=10$). Cyclopentenone mediated monocyte apoptosis was dependent upon serum-withdrawal (2-way ANOVA, Bonferroni post-test, $p<0.0001$, $n=10$).

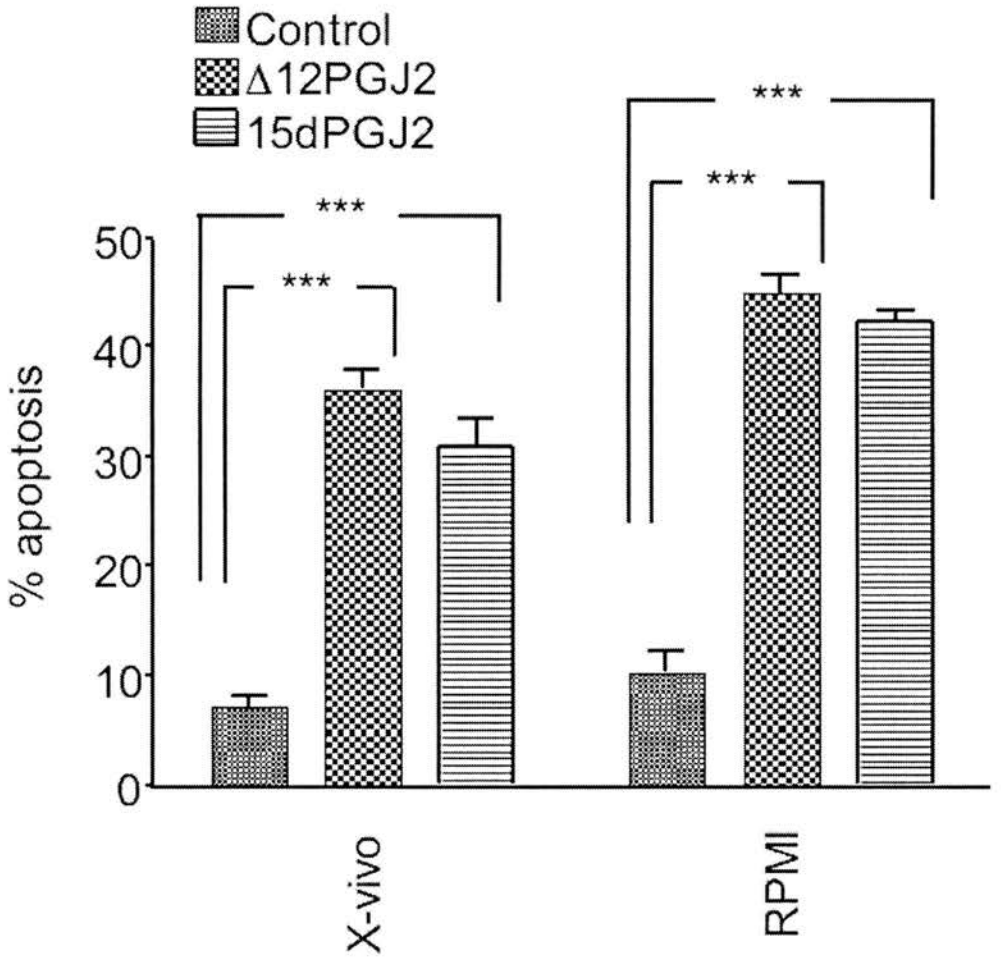


Figure 3-12 THP-1 monocytic leukaemic cell apoptosis, cyclopentenone prostaglandin supplements

THP-1 cells show low levels of constitutive apoptosis in serum-free conditions. Cyclopentenone-induced apoptosis was seen in cells treated at 10μM concentrations for 24 hours. Use of two separate serum-free media, RPMI and X-Vivo 10, produced significant apoptosis induction in serum-free conditions compared to controls (2-way ANOVA, $p<0.0001$, $n=4$).

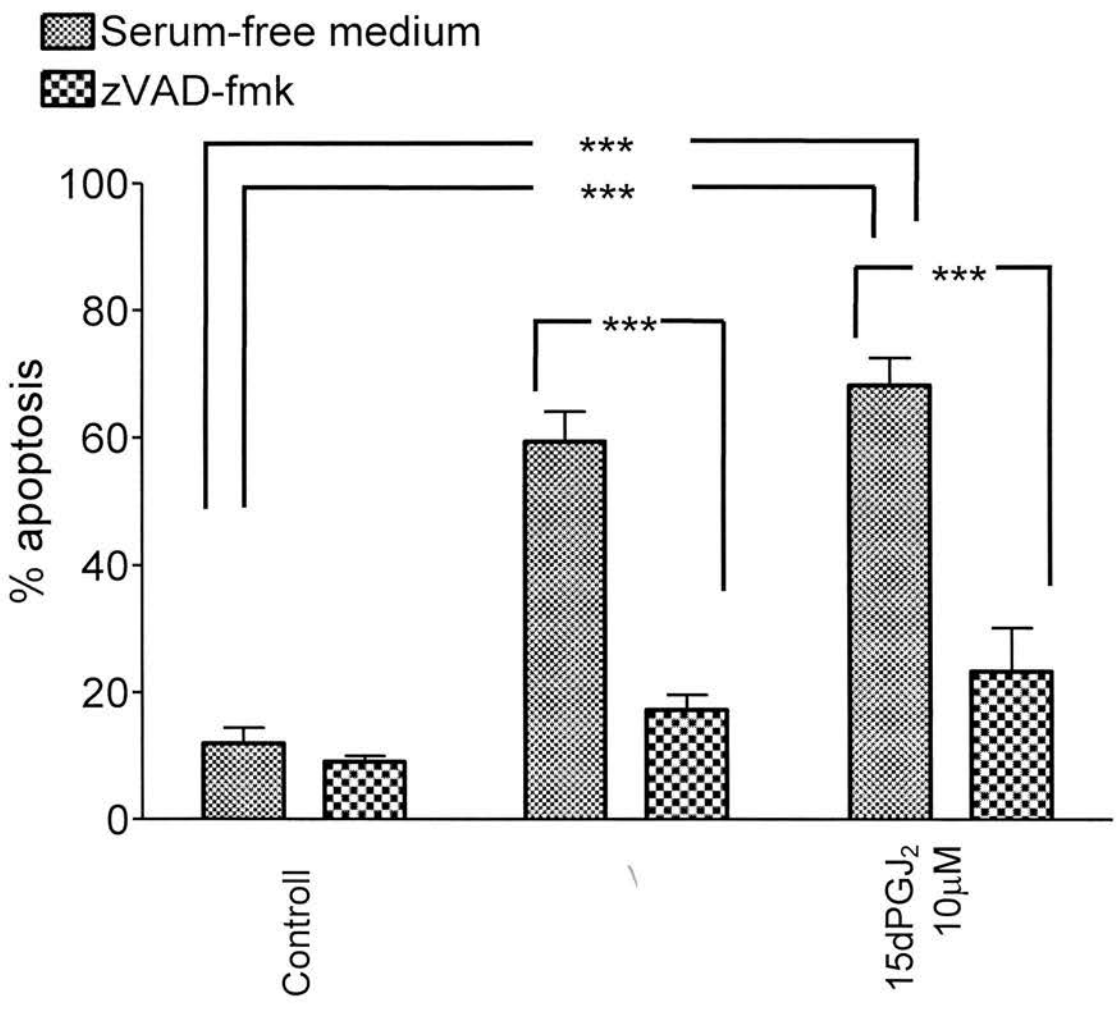


Figure 3-13 Monocyte apoptosis induced by cyclopentenone prostaglandins is partially caspase-dependent

Adherent monocytes cultured in serum-free conditions were pre-treated with the pan-capsase inhibitor zVAD-fmk, before exposure to cyclopentenone prostaglandins at 10μM for 24hrs.
zVAD treatment significantly reduced apoptosis for both cyclopentenone treatments (2-way ANOVA, $p<0.001$, $n=4$). zVAD-fmk did not reduce apoptosis rates to control levels.

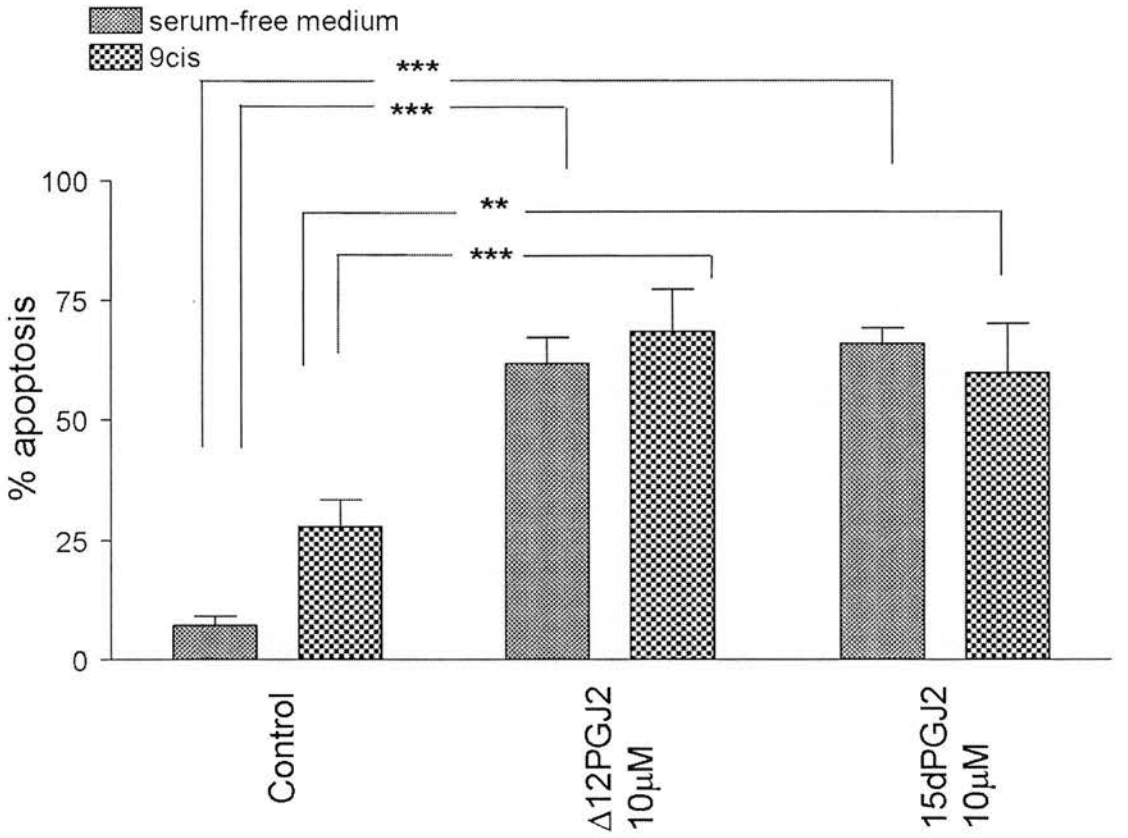


Figure 3-14 Monocyte apoptosis: effects of RXR ligation in conjunction with cyclopentenones

Adherent serum-free monocytes cultured for 24 hours in media supplemented either with cyclopentenone prostaglandins at 10μM alone or cyclopentenones in conjunction with 10μM 9-cis retinoic acid were assessed morphologically for apoptosis. For each treatment, 9(cis) retinoic acid made no significant difference to rates of apoptosis (2-way ANOVA, $p=0.2116$, $n=4$). The cyclopentenone prostaglandins produced significant increases in monocyte apoptosis relative to control ($n=4$, $p<0.001$, except differences between $15dPGJ_2$/9(cis) retinoic acid and 9(cis) retinoic acid alone, $p<0.01$ $n=4$).

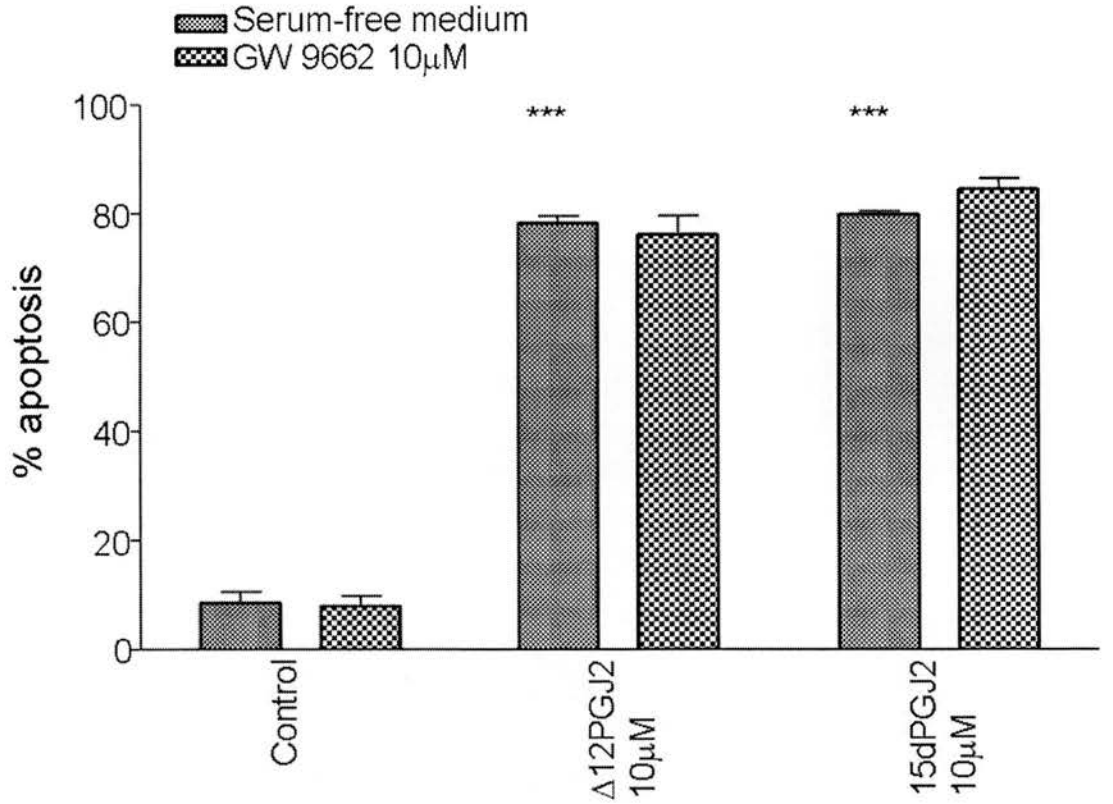


Figure 3-15 Monocyte apoptosis: response to the Glaxo-Wellcome PPARγ antagonist GW 9662 in combination with cyclopentenones

Adherent serum-free monocytes were exposed to the cyclopentenone prostaglandins Δ12PGJ$_2$ and 15dPGJ$_2$ at 10μM for 24 hours with and without the addition of the pharmacological PPARγ antagonist GW9662. Significant levels of apoptosis were induced by the cyclopentenones alone ($p<0.0001$), but this was not altered by co-incubation with GW9662 (2-way ANOVA, n=3, $p=0.7053$).

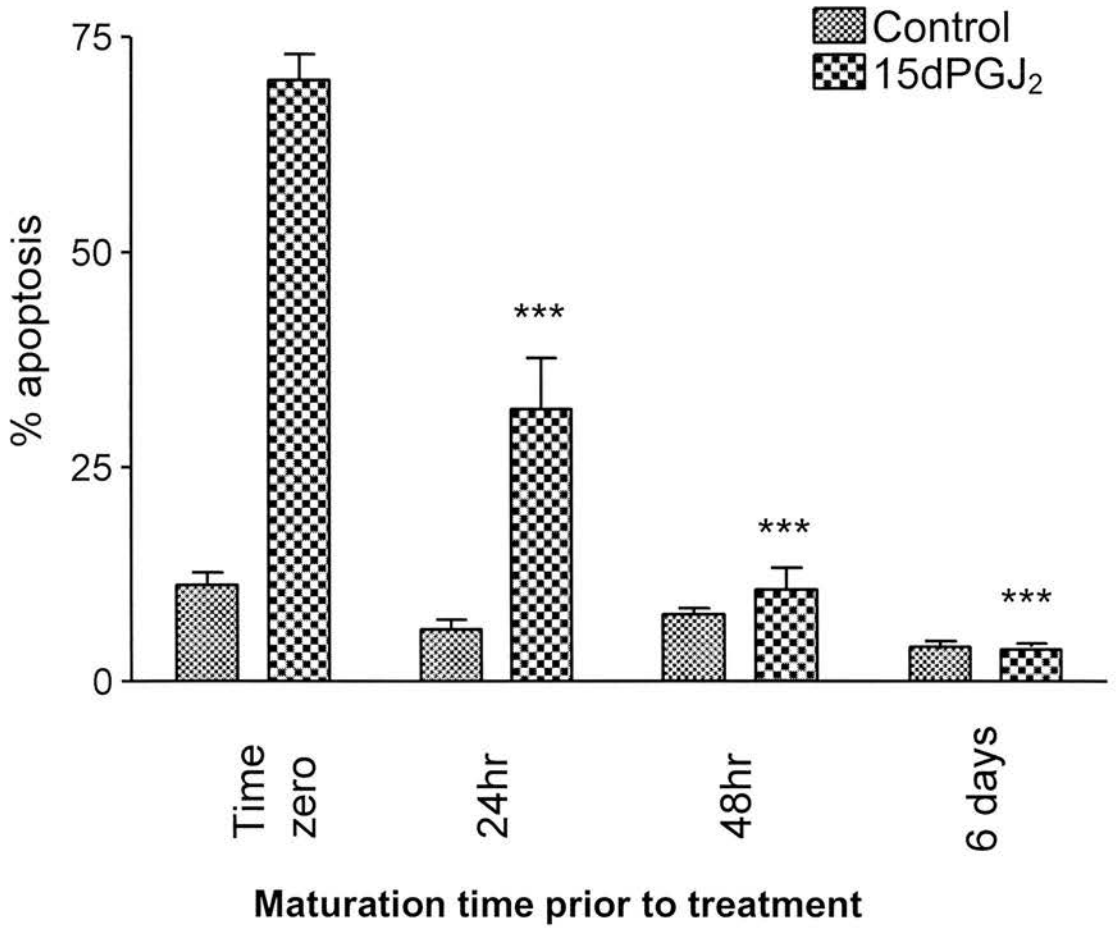


Figure 3-16 Monocyte maturation protects against cyclopentenone-induced apoptosis

Maturation of monocytes for increasing period of time prior to exposure to cyclopentenones conferred protection against apoptosis. Significant reductions in apoptosis were noted after 24hrs, 48hrs and 6 days maturation (2-way repeated measures ANOVA, $p<0.0001$, $n=3$).

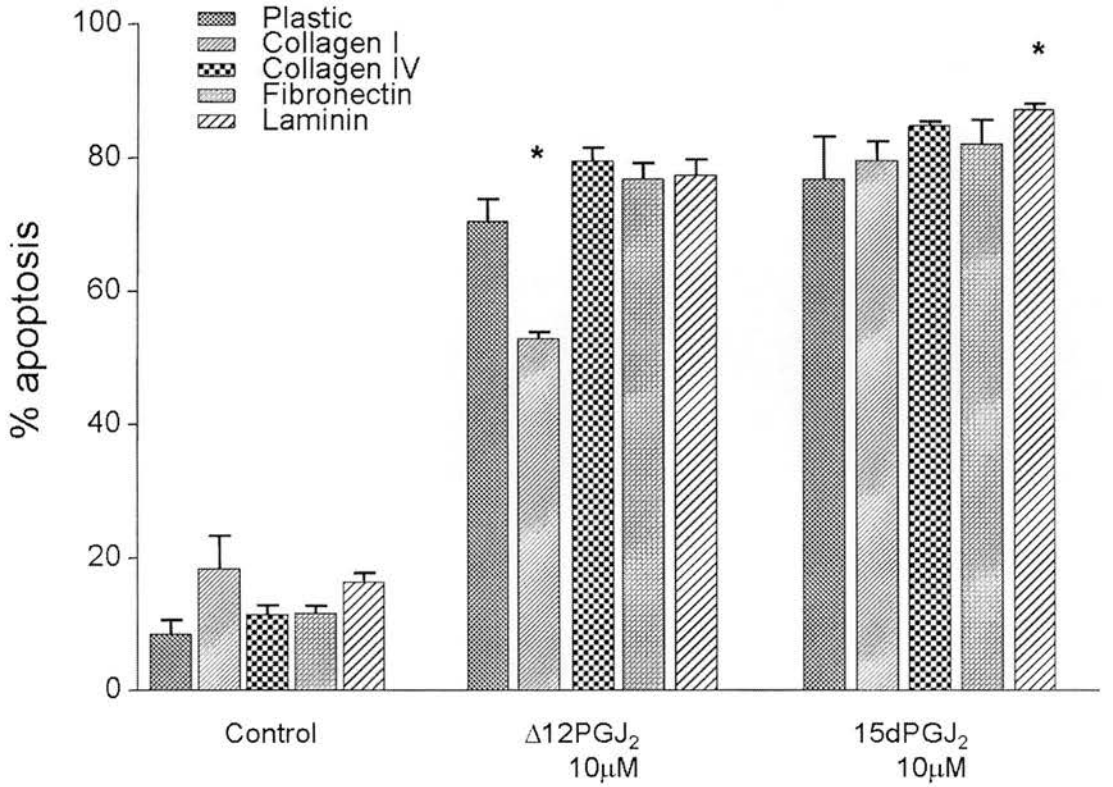


Figure 3-17 Cyclopentenone-induced apoptosis in monocytes adherent to extracellular matrix proteins

Adherence to collagen I reduced monocyte apoptosis induced by $\Delta 12PGJ_2$ (2-way ANOVA, $p<0.01$, $n=4$). Adhesion to other extracellular matrix proteins did not confer protection against cyclopentenone-induced monocyte apoptosis.
Monocyte apoptosis induced by 15dPGJ2 at 10μM was increased in cells adherent to laminin compared to tissue culture plastic (2-way ANOVA, $p<0.05$, $n=4$). This was not reproduced by adhesion to collagen I, collagen IV, or fibronectin.

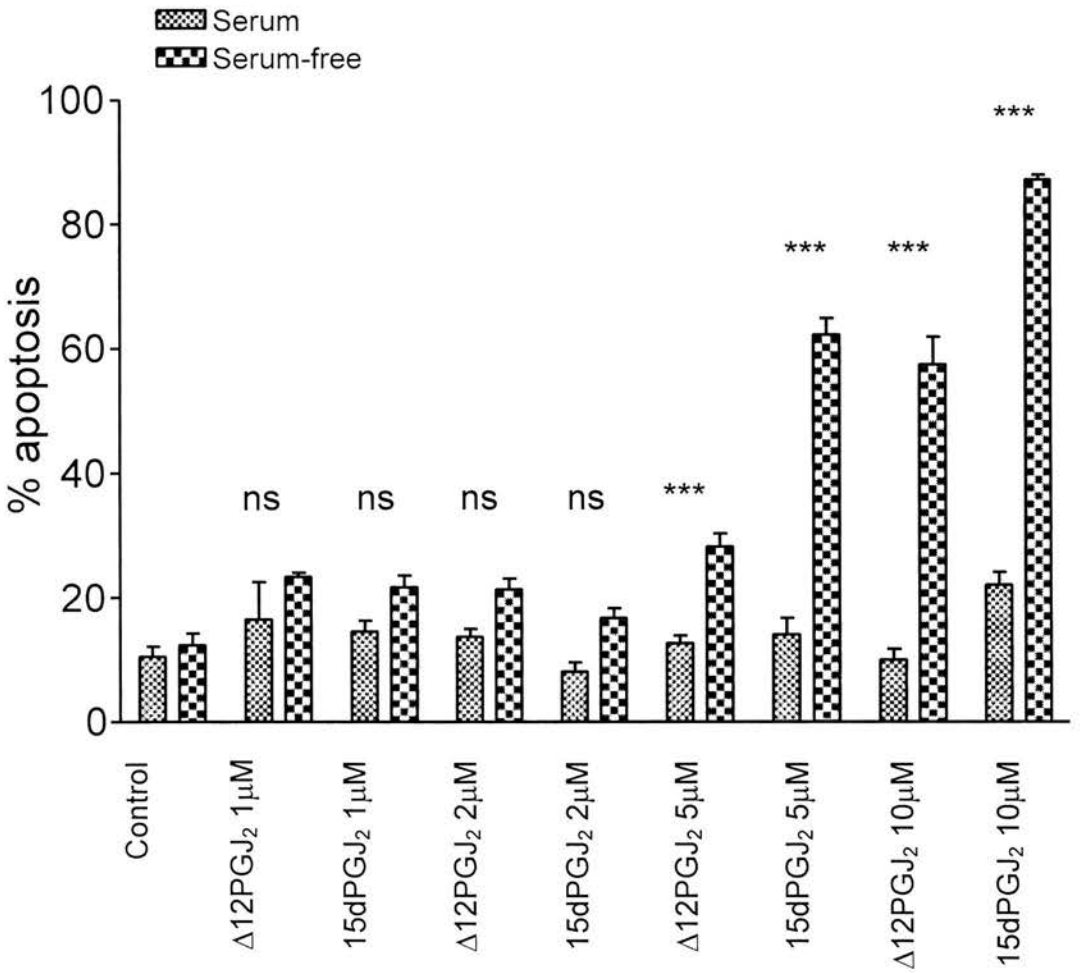


Figure 3-18 Effects of adhesion to fibronectin upon cyclopentenone induced monocyte apoptosis

Mononuclear cells were allowed to adhere to tissue culture plastic coated with fibronectin, before exposure to increasing concentrations of the cyclopentenone prostaglandin ligands $\Delta 12PGJ_2$ and $15dPGJ_2$. Apoptosis was significantly increased in serum-free culture compared to serum-replete culture at cyclopentenone concentrations above 5μM (2-way ANOVA, $p<0.001$, $n=4$).

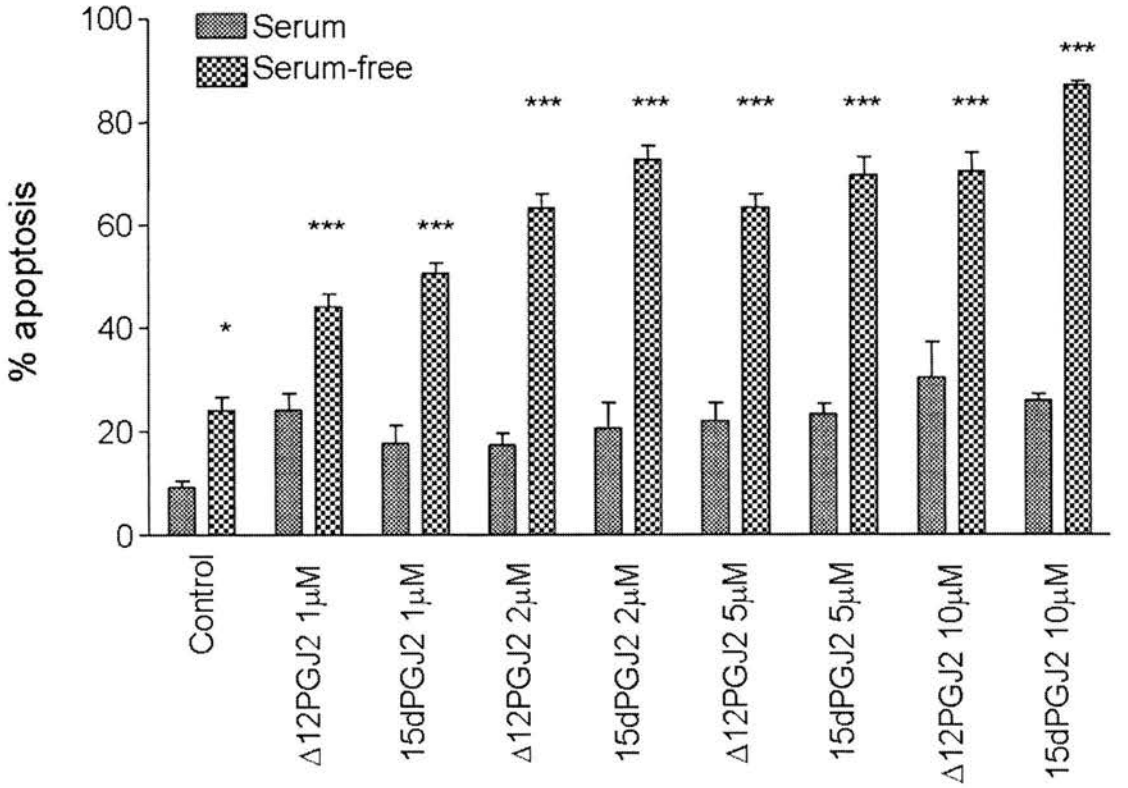


Figure 3-19 Effects of adhesion to laminin upon cyclopentenone induced monocyte apoptosis

Mononuclear cells were allowed to adhere to tissue culture plastic coated with laminin, before exposure to increasing concentrations of the cyclopentenone prostaglandin ligands $\Delta 12PGJ_2$ and $15dPGJ_2$. Levels of apoptosis were elevated in monocytes cultured in serum-free coniditions with control media on laminin relative to tissue culture plastic (2-way ANOVA, $p<0.01$, $n=4$).
Both prostaglandin ligands $\Delta 12PGJ_2$ and $15dPGJ_2$ induced significant levels of apoptosis at all concentrations tested (2-way ANOVA, $p<0.001$, $n=4$).

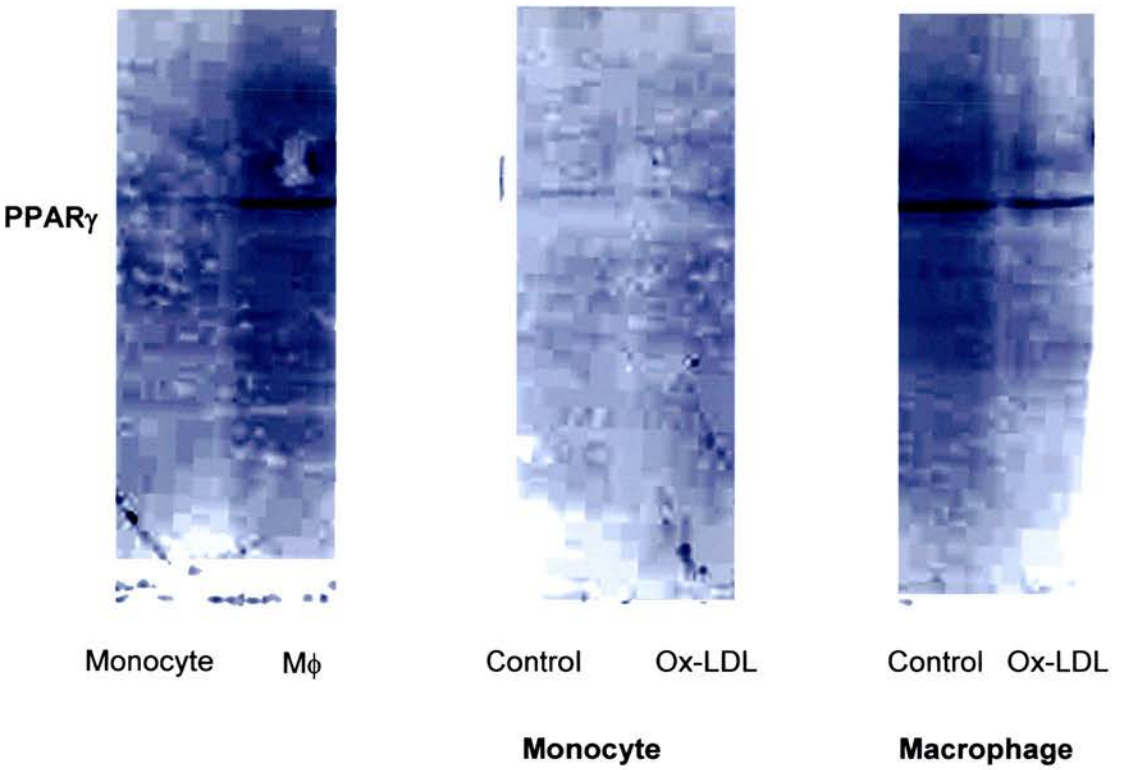


Figure 3-20 Differential PPARγ protein expression in monocytes and macrophages

PPARγ expression, assessed by immuno-blotting whole cell lysates, appeared unaltered in naïve monocytes even after exposure to both acetylated and oxidised LDL at 50μg/ml. However, PPARγ levels were augmented in mature monocyte/macrophages, irrespective of lipid supplementation, or control medium conditions.

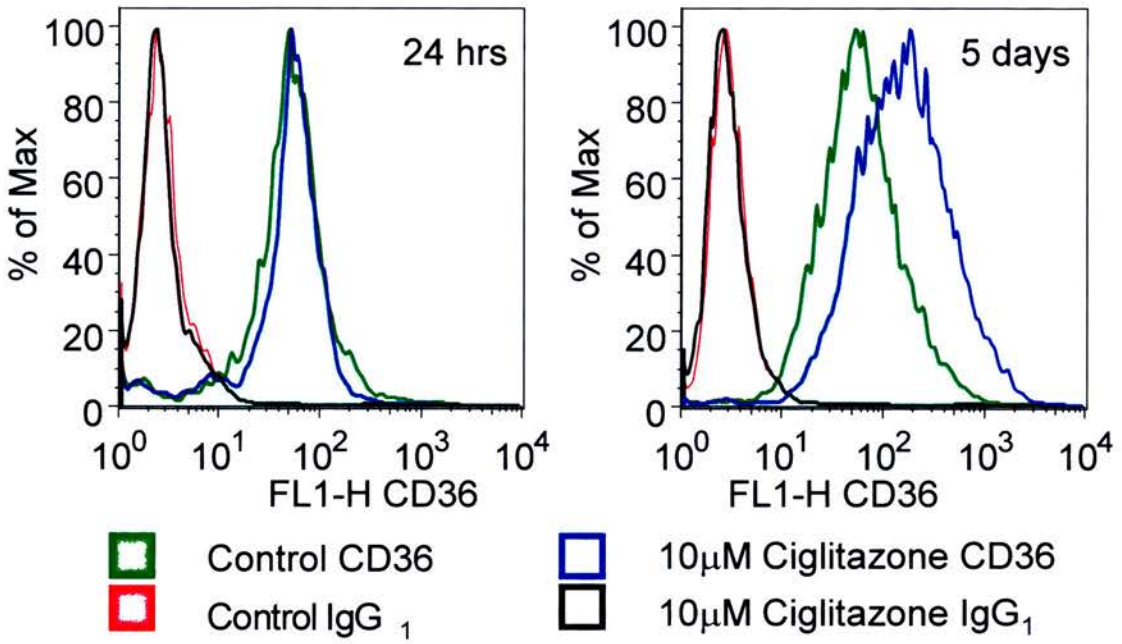


Figure 3-21 Monocyte functional responses to PPARγ ligands are maturation dependent

Primary human monocytes were cultured in suspension, in serum-replete medium. The thiazolidinedione ciglitazone did not enhance CD36 expression in monocytes cultured for 24hrs (left panel). Maturation of monocytes over 5 days followed by ciglitazone stimulation resulted in a visible increase in CD36 immunofluorescence (right panel). (Figure representative of 4 separate monocyte samples).

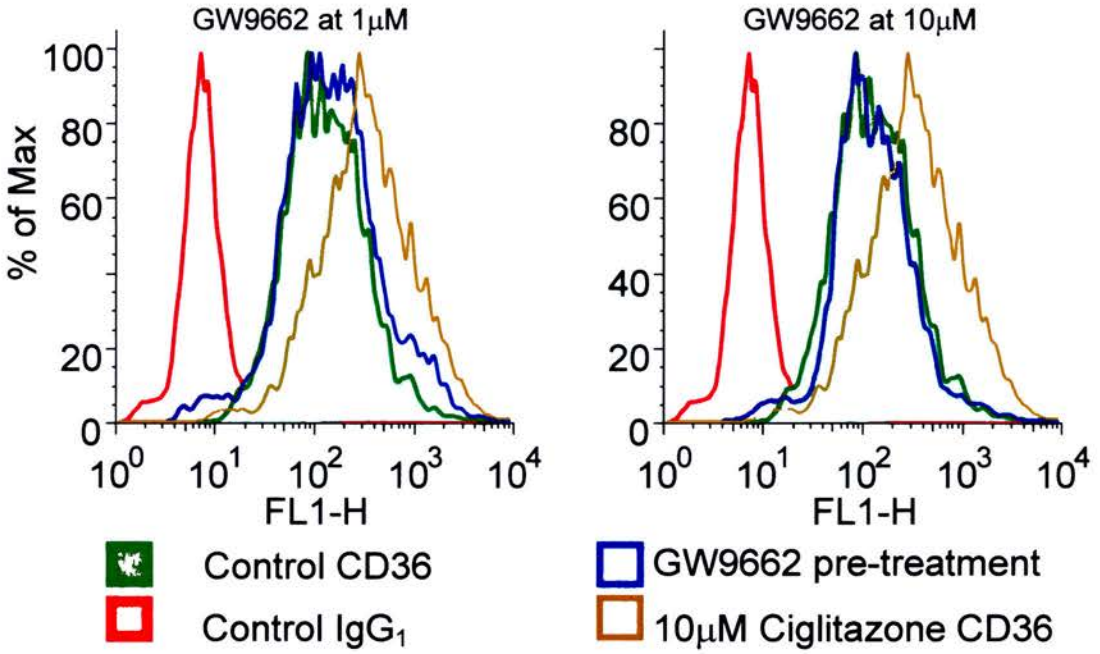


Figure 3-22 Monocyte responses to PPARγ ligands are inhibited by the PPARγ antagonist GW9662

Monocytes were cultured in suspension for 4 days before receiving pre-treatment with the PPARγ antagonist GW9662 at 1μM (left panel) or 10μM (right panel) Cells were then treated with 10μM ciglitazone for a further 24hrs. GW9662 inhibited ciglitazone-mediated CD36 expression at both low and high concentrations (blue histogram overlay). Ciglitazone alone produced an upregulation in CD36 expression (brown histogram overlay). (Figure representative of 4 separate monocyte samples).

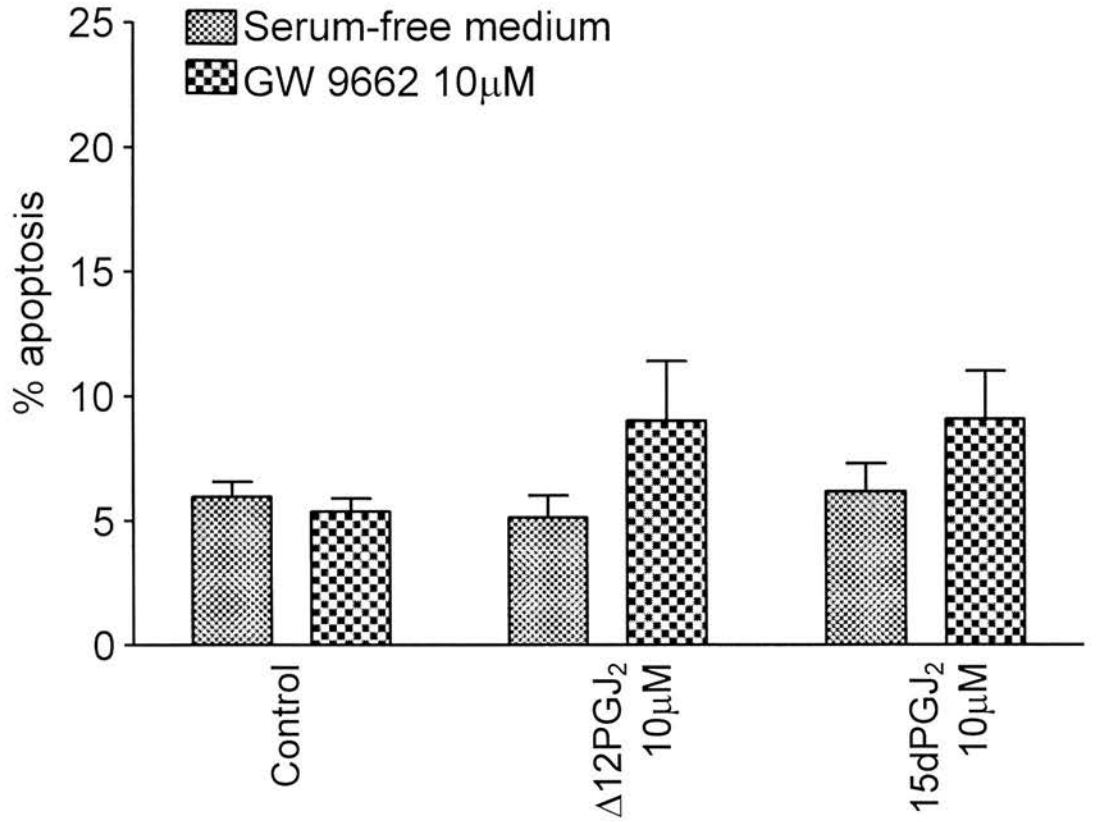


Figure 3-23 Pre-treatment with the PPARγ antagonist GW9662 fails to prevent apoptosis in mature (8 day old) macrophages

Monocytes were isolated and matured by adhesion to tissue culture plastic, in serum-replete conditions. Following pre-treatment with GW9662 for 24 hours on day 6, monocytes were then put into serum free conditions with the addition of cyclopentenone prostaglandins at 10μM for a further 24 hours on day 7. No significant increase in apoptosis was apparent relevant to control (n=4, p=0.1380). No relative change in apoptosis was noted in cells pre-treated with GW9662 (2-way ANOVA, n=4, p=0.4294).

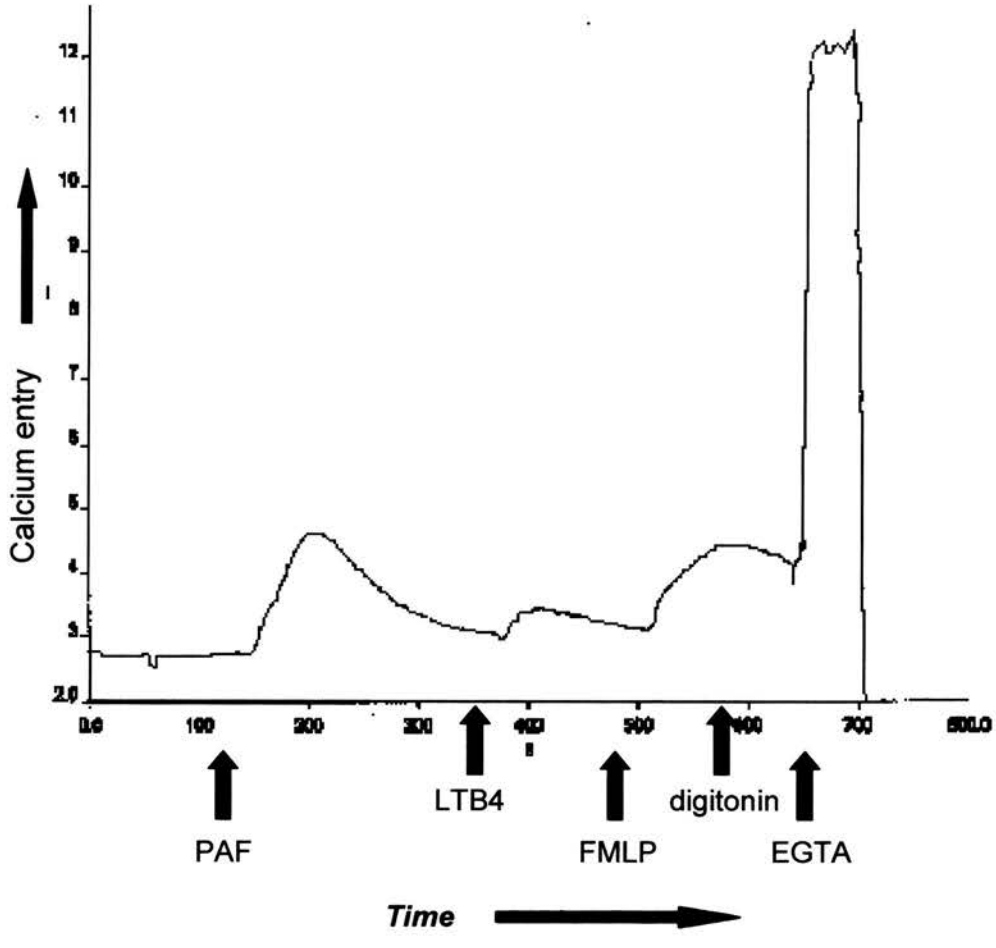


Figure 3-24 Calcium flux is unchanged in monocytes exposed to 15dPGJ$_2$

Freshly isolated un-stimulated monocytes in suspension culture were assayed for calcium flux using a Perkin-Elmer spectrophotometer. Normal responses were seen after exposure to positive stimuli such as fMLP and PAF. Interestingly LTB_4 produced a low level but significant calcium influx. Permeabilisation of cell membranes using digitonin resulted in marked calcium shifts, reversible by specific chelation using EGTA (figure representative of two separate monocyte samples).

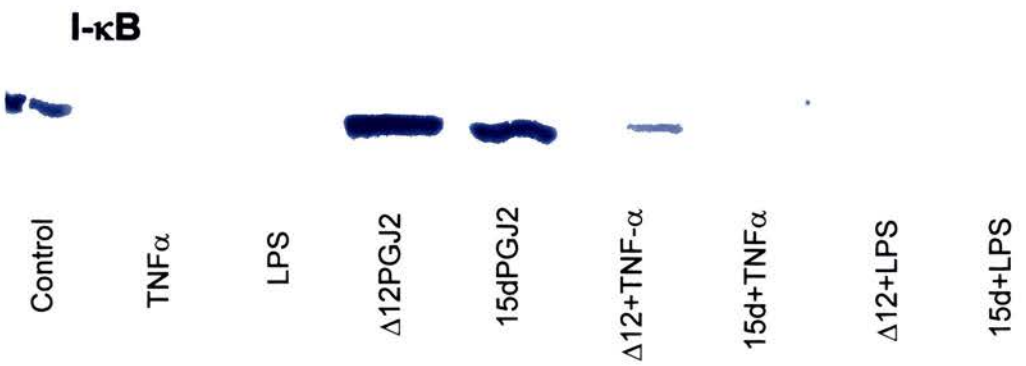


Figure 3-25 Cyclopentenone prostaglandins cause altered NF-κB activation

Immunoblotting for I-κB demonstrated clear bands in control monocytes.
Lysates taken from monocytes stimulated with TNF-α and LPS alone, showed marked degradation in I-κB signals.
Monocytes treated with cyclopentenone prostaglandins displayed a stronger I-κB signal compared to control.
In conjunction with cyclopentenone prostaglandins, TNF-α appeared to only partially induce I-κB degradation, implying a lower level of NF-κB activation in these samples.
In contrast, the effects of LPS were unchanged, with clear loss of the I-κB signal. (Immunoblot representative of three separate experiments).

Chapter 4 USE OF A DOMINANT CO-REPRESSOR PPARγ MUTANT IN THE THP-1 MONOCYTIC CELL LINE

4.1 Introduction

The question as to whether PPARγ directly sub-served apoptosis in monocytes appeared unresolved from the data presented in Chapter 3. The effects that LDL and cyclopentenones have upon monocyte survival might be due to alternate pathways, including the ligation of other PPAR receptors subclasses, such as PPARα and PPARδ. At the time this work was performed, the specificity of GW9662 had not been clarified (Kojo et al. 2003). Although the use of the GW9662 antagonist made the likelihood of a PPARγ-mediated mechanism for monocyte apoptosis induction unlikely, it was felt that further exclusion of the role of PPARγ would help confirm the redundancy of this gene in monocyte survival. One strategy to elucidate the role of PPARγ would be to remove the function of this gene altogether. At the time these experiments were performed, the only practical option to pursue this was the attempted introduction of a mutant PPARγ gene bearing dominant co-repressor activity. This technique had already been performed using a dominant mutant that successfully transfected COS-1 cells (Berger et al. 2000), allowing functional gene silencing. Primary monocytes are notoriously difficult to transfect (Burke et al. 2002). For this reason the THP-1 monocytic cell line was chosen as a target for transfection. The vulnerability of these cells to cyclopentenone mediated apoptosis has already been demonstrated in Chapter 3.

4.1.1 Use of a mutant dominant co-repressor PPARγ construct

To gauge the effects of cyclopentenones upon monocyte apoptosis, and to assess the role, if any of PPARγ in monocyte apoptosis, the introduction of a dominant repressor PPARγ mutant gene was attempted. A dominant mutant PPARγ gene, differing from the one used in COS-1 cells, had been noted to repress PPARγ effects in adipocytes when delivered within the vector pcDNA3. This PPARγ mutant bears two amino acid substitutions that prevent ligand binding and cause co-repression of the wild type gene product (Gurnell et al. 2000)(see Figure 4.1a). Initial attempts to utilise a ligation and complementary nucleotide filling strategy to optimise the introduction of the PPARγ mutant into a fluorescently labelled vector were unsuccessful (Figure 4.2, 4.3 and 4.4). In brief, a "cut-and-fill" strategy was attempted, with the initial use of HindIII to digest pIRES2-GFP. The site of this digest was then to be modified using nucleotides to enable the use of XhoI which would provide a complementary site in pcDNA3. Ligation from this was unsuccessful, and for this reason, a

lengthier but more standardised cloning strategy was embarked upon. pcDNA3 bears multiple cloning sites (MCS) common to pSP72 (Figure 4.1b). This in turn bears common restriction sites to the MCS of pIRES2-GFP (Figure 4.1c). The pSP72 vector was thus used as a shuttle-vector to move the PPARγ insert from pcDNA3 to pIRES2.

Sub-cloning of the construct required multiple manipulations. The PPARγ dominant co-repressor mutant was successfully sub-cloned into the shuttle vector, pSP72, with complementary restriction sites to the original pcDNA3 vector. This latter manoeuvre allowed for subsequent excision and ligation into the GFP intron-containing vector, at the correct site (Figure 4.1c). The strategy of utilising a vector with a GFP intron was adopted to facilitate further selection of successfully transfected cells by flow cytometrically-directed cell sorting.

At each stage of the sub-cloning process, integrity of the ligations was carefully confirmed by restriction digestion and gel electrophoresis. Confirmation of the construct's position within the final vector was performed using restriction digests, at multiple positions. The vector containing the PPARγ mutant was then produced in bulk utilising large scale bacterial plasmid preparations.

4.1.2 Sub-cloning of PPARγ into pSP72

To incorporate PPARγ wild type and mutant fragments into pSP72, the multiple cloning sites were digested using XhoI and XbaI (Figure 4.5), both sites present in pcDNA3/PPARγ and pSP72. The resultant complementary ligation sites enabled the use of pSP72 as a shuttle vector. Difficulties arose with digestion of pSP72, leading at one stage to concern that this vector had been contaminated, or modified. Further digests confirmed the vector to be pSP72, after large scale bulk DNA preparations had been performed (Figure 4.5b). Sufficient quantities of vector and target insert DNA were critical to this process (Figure 4.6), and "maxi" preparations of bacteria were used for DNA production prior to ligations. The use of smaller DNA volumes (Figure 4.7) resulted in unsuccessful ligations (Figure 4.8).

4.1.3 Confirmation of PPARγ/pSP72 construct

Incorporation of the substantial PPARγ fragment into pSP72 produced a high mass DNA product, immediately discernible from vector and insert by gel electrophoresis (Figure 4.9). Confirmation that this was the correct orientation of PPARγ in the vector was undertaken by further restriction digestion using XhoI and XbaI enzymes again, revealing the original vector and insert fragments (Figure 4.10).

4.1.4 Excision of PPARγ from pSP72; insertion into pIRES2

Further manipulations of this shuttle-vector were required to enable the final insertion of PPARγ into pIRES-GFP. Initial attempts were made to use the EcoRI site within pIRES-GFP in conjunction with an XhoI site. This should have produced a simple complementary combination of both PPARγ and pIRES fragments. Complete sequencing details of the PPARγ sequence were not available, and a further undocumented EcoRI site was apparent when an extra fragment was revealed after EcoRI restriction digestion (Figure 4.11). For this reason a process of SmaI and XhoI digests was undertaken for pIRES-GFP and PPARγ/pSP72, as this produced a means of introducing the insert into the vector.

4.1.5 Methylation of pSP72 sites; successful re-derivation of constructs

Despite the use of double and single digests, it proved impossible to produce the correct restriction fragments after enzymatic treatment of PPARγ wt and mutants in pSP72. Variation of restriction digest duration, temperature, buffer pH and the addition or withholding of BSA made little difference. The prospect that a restriction site had been methylated arose, and for this reason it was decided to re-prepare DNA using different competent bacteria. A range of fresh bacteria including TOP10 and GM119 competent cells were utilised, and initially prepared in bulk, before transformation with the pSP72-PPARγ constructs. Interestingly, DNA prepared with these fresh bacterial stocks of both strains now proved amenable to restriction digestion. Serial digests performed first with SmaI and then XhoI produced two clear bands of 2.4kb and 1.5kb, the latter corresponding to the correct length of PPARγ insert (Figure 4.12a). Serial digestion of pIRES2-eGFP with SmaI and XhoI produced a large single visible fragment, as the gap between these sites within the pIRES2 MCS is only 40 bp long (Figure 4.12b).

4.1.6 Confirmation of PPARγ insertion into pIRES2-GFP

Confirmation of correct insertion of the PPARγ insert after this final ligation was performed by cutting the new construct with BamHI. This produced two large visible (5.9kb and 1kb) fragments as well as a small (9bp) fragment that was obviously not seen on these gels. These digests were confirmed and also cross-checked with digests of the original vectors and the shuttle vector, to ensure that no hidden sites had been unaccounted for. Initial BamHI digests were performed to identify successful ligation products: DNA mini-preparations were made from colonies of bacteria transformed with PPARγ/pIRES2 ligation product (Figure 4.13). Further restriction digests of all the vectors used at each stage of cloning were then performed, checking fragment lengths against restriction maps. Fragment lengths were

appropriate for both mutant and wild type PPARγ, and the original vectors showed no evidence of other hidden BamHI sites (Figure 4.14). This meant that PPARγ had successfully been incorporated into pIRES2-GFP, enabling the use of this construct to transfect cells with plasmid DNA resulting in the expression of a fluorescent protein marker.

4.1.7 Transfection of THP-1 cells with pIRES2/PPARγ

Transfection of THP-1 monocyte cell lines was attempted. Standard lipofection with Lipofectamine (Invitrogen USA) proved unsuccessful, with no cells showing any GFP expression (data not shown). Electroporation of the pIRES-GFP/PPARγ construct was then attempted. This proved to be a significant technical challenge, with multiple modalities of transfection resulting in no evidence of successful transfection. Utilisation of novel electroporation protocols with varied pulse times, and pulse morphologies, including biphasic pulse waveforms (Table 4.1) were also unsuccessful (EquiBio double pulse electroporation and Amaxa Nucleofector protocol, GmBH, Figure 4.15).

THP-1 cells were pre-treated in ATP-supplemented buffers, prior to electroporation using the EquiBio double and single pulse systems with vector DNA. Cells were allowed to recover from electroporation for 48hrs in culture before flow cytometric assessment. Apparently successful transfection was documented by fluorescent microscopy (Figure 4.16). Double-pulse waveforms, although supposedly more efficient in effecting transfection, produced little scatter shift with sham electroporations or with wild-type/vector constructs. Furthermore, sub-gating on these sub-populations revealed no noticeable increase in green fluorescence in the FL1 channel (Figure 4.17). No changes in fluorescence were noted with double-pulse electroporation using the pIRES/PPARγ mutant construct, even when sub-gating on the higher side-scatter population (Figure 4.18). Single-pulse electroporation caused a marked increase in THP-1 cell side-scatter (Figure 4.19). No noticeable increase in fluorescence was seen in the high side-scatter gate when comparing sham electroporation with wild-type/vector constructs. Similar results were seen with single-pulse transfections using the pIRES/PPARγ mutant construct (Figure 4.20), again with no noticeable difference in green fluorescence between sham electroporations, and DNA-loaded electroporations. This suggested that despite the fluorescent microscopy data being encouraging, the visualised increase in green fluorescence was an artefact of electroporation. A proportion of the high side-scatter electroporated cells appeared to have condensed nuclear morphology on cyto-centrifuge preparations, suggesting reduced viability post-transfection (data not shown). Use of the Amaxa nucelofector (Amaxa GmBH) with nocodazole supplements to hold cells in G_0 phase was also unsuccessful, (data not shown). The pIRES-GFP vector had already

been used to transfect COS-1 cells using similar protocols, demonstrating competence of both vector and electroporation (A. Lacy-Hulbert, pers. comm. 2002). The PPARγ mutant had also been successfully been transfected into COS-1 cells (Berger et al. 2000). Unfortunately it was not possible to produce any successful transfections of THP-1 monocytes using the PPARγ construct, and thus the full range of planned viability assays was unable to be performed.

4.2 Summary

THP-1 cells were used as a target for the introduction of a dominant mutant PPARγ gene construct.

A mutated PPARγ sequence, with documented repressive functions in adipocyte models, was successfully manipulated through a series of plasmid vectors.

Despite problems with restriction enzyme digestion due to methylation of digestion sites, the mutant gene was inserted into the multiple cloning site of a pIRES bicistronic vector, which enabled target cell expression of GFP.

Lipofection with pIRES/PPARγ was unsuccessful.

Electroporation of THP-1 monocytic cells was attempted using two electroporators, varying electrical pulse width and morphology. To optimise transfection, cells were held in cell cycle arrest. Despite varying parameters, transfection of THP-1 cells proved not to be reproducible.

4.3 Discussion

4.3.1 Molecular manipulations of plasmid DNA

Introduction of GFP expressing vectors into eukaryotic cell lines is well described (Rees et al. 1996). The use of vectors with multiple cloning sites is an established means of introducing genes of interest into such vectors, allowing the tandem expression of a fluorescent marker indicating successful translation of the protein (Cormack, Valdivia, & Falkow 1996). The difficulties in introducing a mutant gene sequence into the pIRES vector were focused at the level of molecular manipulation. Methylation of a critical digest site in one of the vectors used to sub-clone the PPARγ mutant resulted in a lengthy delay in progress, and necessitated multiple attempts to re-digest the vector involved (Kessler, Neumaier, & Wolf 1985). This particular hurdle was overcome by re-deriving the shuttle vector, and involved freshly transforming competent bacteria, and performing a further plasmid preparation. This restored the shuttle vector cloning site, with restriction enzyme sites that were amenable to digestion, facilitating the successful introduction of the PPARγ mutant into the pIRES vector.

4.3.2 Gene transfection

Gene transfection into mammalian cells involves the introduction of nucleic acids across the cell membrane, with subsequent trafficking to the nucleus (reviewed in Burke et al. 2002). There are significant obstacles to such a process, including the electrostatic repulsion between negatively charged nucleic acids and the negatively charged outer membrane of target cells. Plasmid DNA size is limited in terms of entry to 15kb by membrane pore size, requiring condensation to enable entry into cells. DNA entering into the cytoplasm is degraded by endosomal nucleases, limiting the ability of exogenous DNA to reach the nucleus.

4.3.3 Difficulties with electroporation

Electroporation involves passing an electric pulse through a suspension of cells and exogenous DNA. Exposure of eukaryotic cells to electric fields is thought to alter membrane structure, facilitating the transient formation of pores that allow the entry of exogenous nucleic acids. DNA is believed to move directly into the nucleus of electroporated cells, bypassing endosomal degradation pathways that would otherwise destroy exogenous nucleic acids. The success of this technique is critically dependent upon individual factors including the potential difference and capacitance, ionic strength and temperature of the medium, and

the quantity of DNA used. To ensure robust transfections, these factors have to be optimized for each cell type (Weir & Meltzer 1993).

The electroporation techniques described in this chapter are an adaptation of a method successfully used to transfect a GFP expressing vector into J774 macrophage cell lines, to explore functional phagocytic responses (Smits et al. 1999). Given that this group was able to produce an efficient transient transfection, with sufficient cell recovery and viability to then assay macrophage function, it was hoped that a similar outcome would occur for THP-1 cells. The position that individual target cells occupy within the cell cycle is also of critical importance. Successful transfection is affected by the proportion of cells in S phase, a factor of particular importance in the transfection of granulocyte-macrophage progenitors by electroporation (Takahashi et al. 1992). In an attempt to maximise DNA entry to THP-1 cells, alterations in pulse duration, intensity and pulse type were used. The failure of double-pulse waveforms to successfully transfect THP-1 cells was surprising. Double-pulse waveforms produce a transient depolarisation at the cell membrane, followed by a longer duration electric pulse permitting the passage of larger amounts of DNA into the cell across a gradient of potential difference (Atkins, Wang, & Burke 2000).

Nucleofection, a refinement of electroporation that has been reported to improve DNA delivery to the nucleus has been reported as successful in monocyte and macrophages (Martinet, Schrijvers, & Kockx 2003). Nucleofection relies on pulse forms that optimise DNA trafficking into the nucleus, bypassing cytoplasmic trafficking and degradation of nucleic acids. Nucleofection is more efficient if cells are quiescent or held in cell cycle arrest (Trompeter et al. 2003). Nucleofection was thus performed in the presence of nocodazole, a cell cycle inhibitor. However, a transfection strategy that arrests cell cycle is perhaps not the optimal means to examine cell survival. Further exploration of this technique was limited by time constraints.

Electroporation has been reported in monocyte-derived macrophages *in vitro* (Weir & Meltzer 1993), but is poor in pro-monocytic/leukaemic cell lines with reported success rates of less than 3% (Liao et al. 1997). Electroporation produces high levels of cell death, ranging from 5% to 60% in cell lines within 72 h after electroporation (Kusumawati et al. 1999), to 30 to 75% within 24 h using primary cells (Weir & Meltzer 1993). In practice, it appears that lower levels of cell death are associated with lower transfection success rates. CD34+ endothelial progenitor cells are mononuclear cells that have been successfully electroporated by pelleting cells following pulse delivery, and then incubating them in the caspase inhibitors B-D-fluoromethyl ketone and z-VAD-fmk, a procedure that reduced osmotically induced swelling, limiting cell death and produced transfection efficiencies of 20% (Li,

McCarthy, & Hui 2001). However this has not been reported in primary human macrophages. Electroporation thus still appears to be a technically difficult *in vitro* mode of transfecting primary monocytes and macrophages.

4.3.4 Further transfection options

Transfection of primary cells is technically demanding, with monocyte/macrophages being notoriously resistant to transfection methods (Burke et al. 2002).

Methods of DNA delivery to primary cells other than electroporation include lipofection methods direct infection with viral vectors, and more rudimentarily the attempted introduction of naked DNA.

4.3.5 Naked DNA delivery to macrophages

Macrophages can take up exogenous DNA, possibly *via* specific transport mechanisms. However, most extrinsic DNA is degraded in endosomes (Bennett, Gabor, & Merritt 1985). However, high concentrations of exogenous DNA have been successfully used to transfect the RAW 264.7 macrophage cell line (Stacey, Sweet, & Hume 1996). Studies on human dendritic cells have shown the successful transfection of naked DNA into cutaneous DCs in human subjects (Condon et al. 1996), although this latter approach was non-specific in terms of target cell uptake of DNA. However, a significant drawback of naked DNA transfection is the presence of unmethylated CpG dinucleotides (cytosine followed by guanine), which are rare in eukaryotic DNA. These sequences induce macrophage activation similar to that caused by LPS, because unmethylated CpG DNA is a potent adjuvant (Krieg 1999). This makes naked DNA transfection highly impractical for macrophage work.

4.3.6 Liposome DNA delivery and cationic compounds

Enhancing DNA transport through the cell membrane and into the nucleus, requires a variety of complex-forming and condensing agents (reviewed in Zhdanov, Podobed, & Vlassov 2002). Polyvalent cations including calcium and manganase, polycations (such as spermine, spermidine, histones, polyethylenimines), cationic polypeptides and dendrimers and cationic liposomes may all be used. These transfection mediators aid condensation of DNA. The resultant nucleic acid compaction promotes penetration into the cell, and protects DNA integrated into condensed complexes from nuclease-mediated degradation. Gene delivery with liposomes (lipofection) is the commonest current mode for DNA delivery to eukaryotic cells, offering low toxicity, and permitting delivery of variable sizes of DNA fragments (Felgner et al. 1994).

Attempts at using lipid-based reagents to transfect primary macrophages or myeloid cell lines *in vitro* have produced poor transfection efficiencies (<5%), with transgene expression lasting no more than 24hrs (Kusumawati et al. 1999). Lipofection was attempted with THP-1 cells, but was totally unsuccessful, and these data were not presented. The use of DEAE-dextran to counter-balance negative charge effects is another means of facilitating DNA transfection in mammalian cells (Danna & Sompayrac 1982) and has been used as a DNA transfection method for adherent primary human macrophages, with improved transgene expression of up to 56 h (Mack et al. 1998). However, this can alter residual monocyte survival and was avoided. A variety of commercial liposomal agents such as LipofectAMINE (Invitrogen USA), combining liposomes and DNA with the DNA-condensing agent protamine sulphate, have been shown to successfully transfect the murine macrophage cell line RAW 264.7 (Dokka et al. 2000), although success was determined by luciferase activity for whole culture wells, thereby exaggerating transfection success rates. The use of GFP-reporter sequences is a more accurate method, offering the ability to distinguish individual cell transfection success, and was a key factor in the choice of vector used to perform the THP-1 transfections presented in this chapter.

4.3.7 Adenoviral strategies

Adenoviral vectors have been used successfully to transducer monocytes and macrophages, both in culture lines and in primary cells. Macrophage activation may be down-regulated following adenoviral delivery of suppressors of inflammation including small protease inhibitors (Henriksen et al. 2004) and inhibitors of NF-κB degradation including dominant negative I-κB mutants (Wilson et al. 2005). However, adenoviral vectors not bearing similar anti-inflammatory genes introduce the possibility of macrophage activation (Lieber et al. 1997). As discussed in chapter 3, this is a potentially anti-apoptotic stimulus and was thus avoided for these experiments. Adenoviral-mediated introduction of the same PPARγ mutant used in this chapter has been successfully performed in adipocytes (Nugent et al. 2001). Discussion with this group suggested that attempts at use of an adenoviral vector for delivering PPARγ to macrophages had indeed produced target cell inflammatory responses (Agostini M., Gurnell M., pers. comm. 2002) precluding the use of this vector for apoptosis studies.

4.3.8 Microorganisms

Microbial entry into host cells is a feature of successful pathogens, and has been exploited for DNA delivery into mammalian cells. Human monocyte-derived macrophages have been

successfully transfected with *Listeria monocytogenes* (Dietrich et al. 1998) and *Salmonella typhimurium*, with transfection efficiencies in the latter system of 85–95% (Paglia et al. 1998). However, as this is likely to invoke pro-inflammatory and pro-survival responses this was not a useful strategy.

4.3.9 Receptor-mediated gene transfer

Macrophage surface phenotyping has fostered the development of specific targeting routes to permit DNA transfection. By exploiting endocytosis to facilitate DNA entry, mannose has been incorporated into complexes with DNA and used to increase transfection efficacy for primary *in vitro* macrophages (Ferkol et al. 1996). Integrins may also serve as a target to aid DNA entry into macrophages. The use of Ligand Integrin DNA vector systems has been reported to increase transfection efficiency in conjunction with lipofectin (Hart et al. 1998). However, a major concern in this work was the use of monocytes which had not undergone prior activation before exposure to pro-apoptotic stimuli. Adhesion-dependent cell survival is a principal feature of integrin mediated interactions with extracellular matrices (Giancotti & Ruoslahti 1999). The anti-apoptotic programmes that may be initiated by adhesion events were discussed in Chapter 3. β1 integrin ligation is particularly relevant in this regard. Integrin-linked kinase associates with β1 integrin in the focal adhesion complex, and has been recently shown to mediate survival *via* a PKB/Akt mediated signalling pathway (Nho et al. 2005). This suggests that receptor mediated transfection is a less than ideal strategy for gauging subsequent cell death responses.

4.3.10 Alternate strategies to silence genes

Introduction of exogenous proteins into cells may effect inhibition of cell function avoiding the need for gene transfection. A useful tool in this regard is the Tat protein system, fusing proteins of interest with a 36-amino acid sequence from the human immunodeficiency virus protein Tat (Becker-Hapak, McAllister, & Dowdy 2001). However, this was not appropriate for the introduction of the PPARγ dominant suppressor gene sequence.

A more realistic strategy to selectively silence PPARγ would be the use of small interfering RNA (siRNA) sequences. Uptake of short sequences of double stranded RNA results in the degradation of homologous sequences of mRNA in host cells, offering the ability to suppress (or "knock down") functions of specific genes (Elbashir et al. 2001). Tailored sequences of siRNA mimic this natural method of gene silencing, and have been successful in inhibiting PPARγ mediated maturation and differentiation in pro-adipocytes (Hosono et al. 2005).

Use of PPARγ null animals has enabled issues of incomplete pharmacological antagonism or ineffective gene silencing to be avoided. PPARγ is essential for placental and cardiac growth, making PPARγ gene deletions embryonic lethal (Barak et al. 1999). However, work using PPARγ-null embryonic stem cells has allowed the use of embryoid bodies which have been successfully differentiated into macrophages, enabling further study (Chawla et al. 2001b). Further work has been performed using the Cre/LoxP system of conditional gene deletions to target tissue specific removal of the PPARγ gene, and this latter strategy may be of most use in further monocyte and macrophage work (Akiyama et al. 2002).

4.3.11 PPARγ specific responses in monocytes

Gene suppression may be necessary in elucidating the dependence of particular monocyte responses upon PPARγ. Understanding of the intra-cellular handling of cholesterol by macrophages has been aided by the use of PPAR-null cells. The key issue that led to this part of the project was the prospect that the PPARγ antagonist GW9662 might be either non-specific, or lose efficacy during experiments. Subsequent data has shown that GW9662 is highly PPARγ specific, and the data in Chapter 3 showed this agent to be functional.

Monocytes and monocytic cell lines are difficult to transfect, without altering cell viability or activation status. The individual vectors and electroporation techniques had been used successfully in other cell culture systems, but were not reproducible in THP-1 cells. In retrospect, the attempt to silence PPARγ may not have been necessary. However, such gene strategies are often necessary to clarify the complex interdependence of regulatory genes in cell systems.

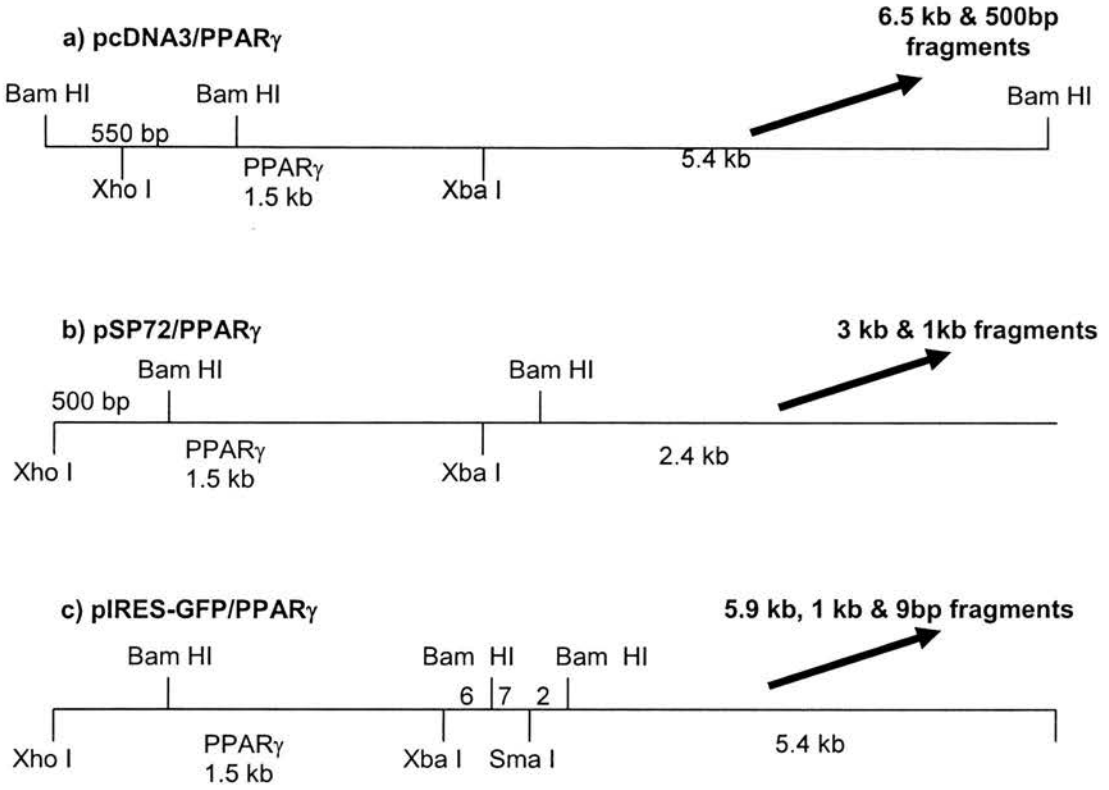


Figure 4-1 Cloning strategies

Strategies used to introduce PPARγ dominant mutant into GFP-expressing pIRES vector are displayed according to the restriction maps of the vectors used.

The multiple cloning sites for individual vectors are shown. For each vector, relevant restriction digest sites used for sub-cloning are shown and fragment sizes are indicated.

Figure 4-2 Attempted construction of bi-cistronic vector

An attempt was made initially to digest the pIRES-GFP vector with HindIII (a), and then fill nucleotide sequences enabling subsequent digestion with XhoI. This would have produced a complementary site for ligation. The PPARγ wild type (b) and mutant (c) were digested with XbaI and then XhoI to produce ligation-ready sites. Ligation was performed using DNA ligase I with a 3:1 insert:vector molar ratio. (Lane 1 shows a 500bp molecular weight marker ladder, lane 2 shows a low DNA mass ladder. Vector mass was estimated as 50-100ng using the mass ladder.)

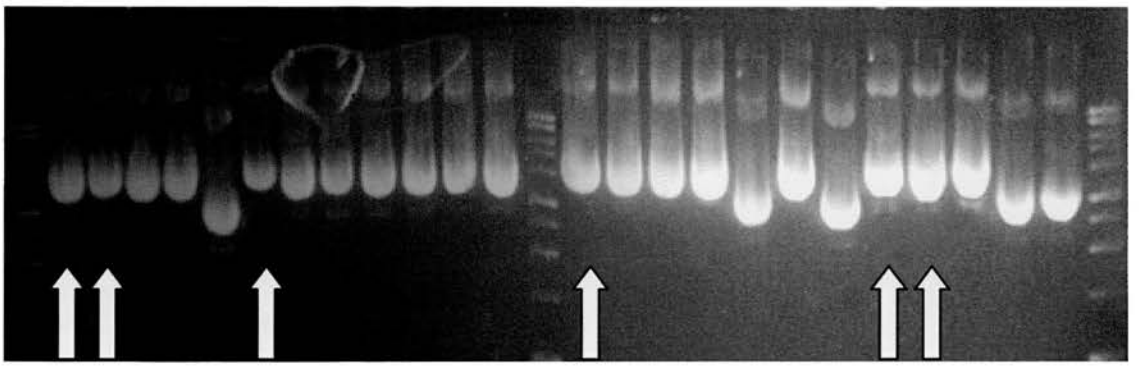


Figure 4-3 Plasmid preparation of ligated products

Ligation products were transfected into competent bacteria and DNA mini-preparations used to produce DNA samples for confirmation of ligation success. Six random plasmid preps producing large products were picked (arrows). Samples were divided into wild-type (lanes 2-13) and mutant (lanes 15-27). Molecular weight markers intervene.

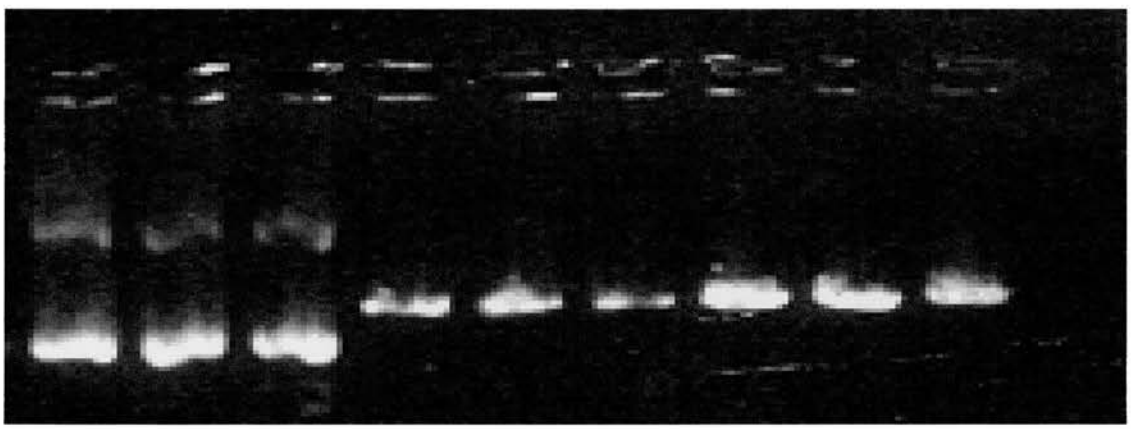


Figure 4-4 Failure of ligation using initial methods

Plasmid products were digested using BamHI as this would produce two large products visible on electrophoresis. Unfortunately, only linearised pIRES-GFP vector appeared, suggesting that ligation had not occurred using a "cut/fill" strategy to produce complementary sites.

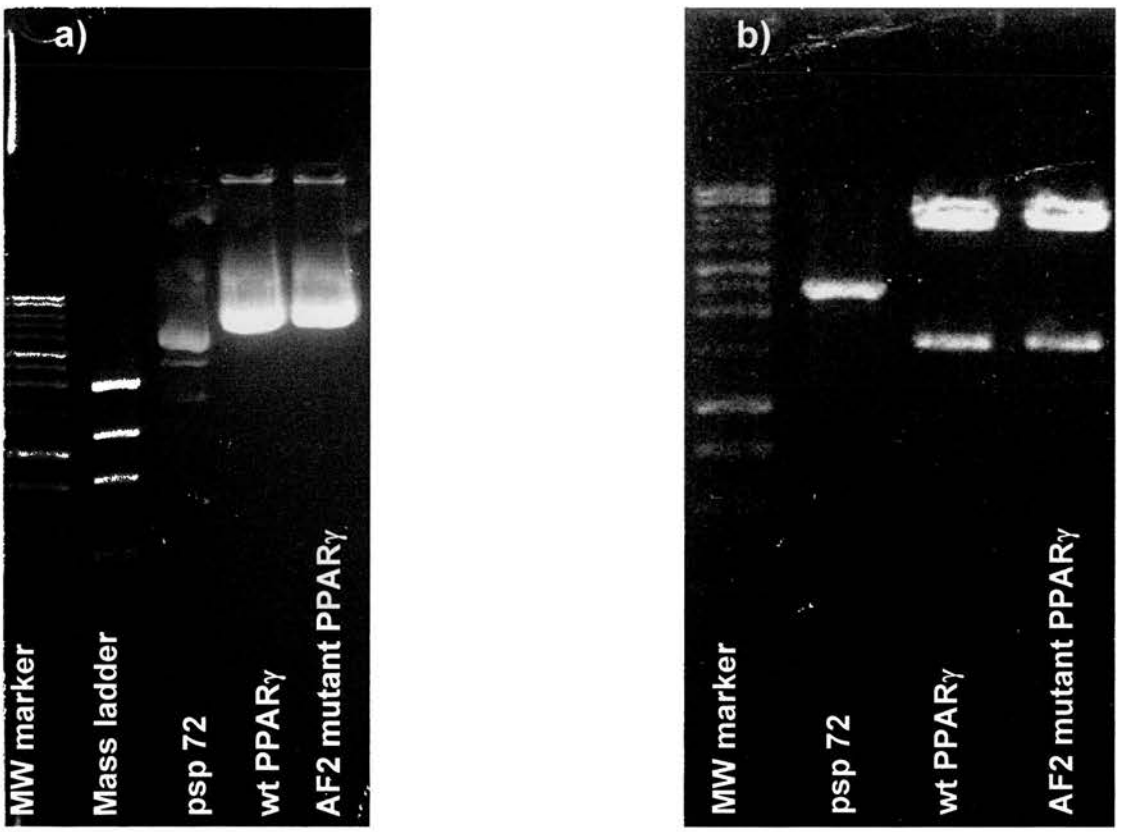


Figure 4-5 Use of psp72 as shuttle vector

The vector psp72 bears complementary restriction sites to the pcDNA3 vector. Insertion of the PPARγ construct into psp72 leaves a further restriction site complementary to sites in the pIRES-GFP vector. Figure 4.5a shows wild type PPARγ, and AF2 mutant run against 1 kb and DNA MW ladder; Figure 4.5b shows fragments after successful restriction digestion with XhoI and XbaI.
psp72 has linearised, but only one cut fragment is large enough to be visualised. Both PPARγ constructs have cut appropriately, with two visible fragments for both.

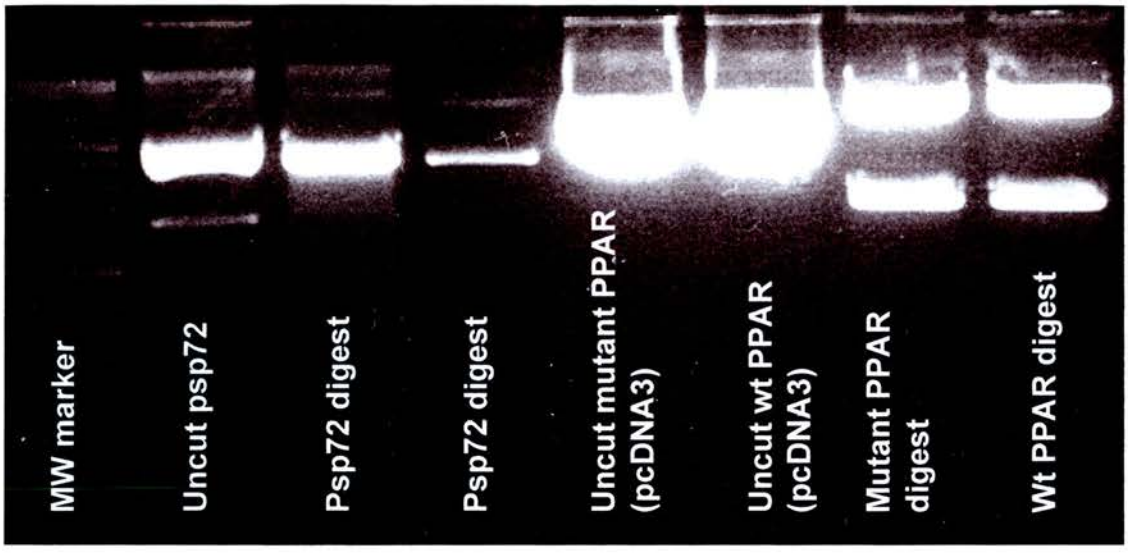


Figure 4-6 Gel purification of XhoI/XbaI fragments.

PPARγ-containing pcDNA3 and empty pSP72 vectors were simultaneously cut with XhoI and XbaI to produce fragments for ligation. Sufficient masses of DNA were used to enable gel purification from these fragments.

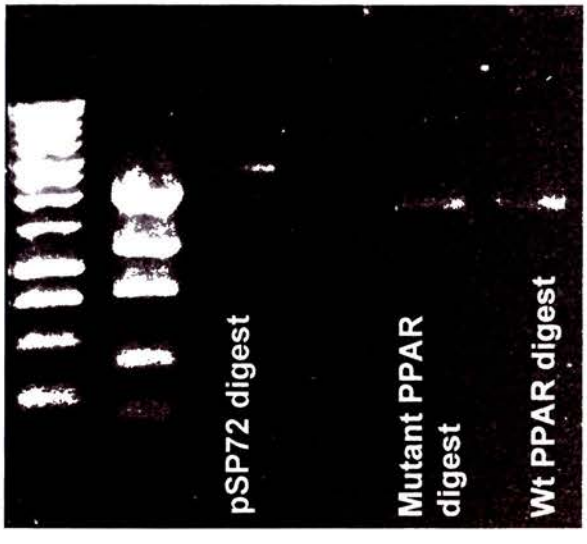


Figure 4-7 Digest products, post-purification

(Lane 1, 1kb molecular weight ladder; Lane 2, low DNA mass ladder). Gel purification gave low yields, prejudicing the success of subsequent ligations.

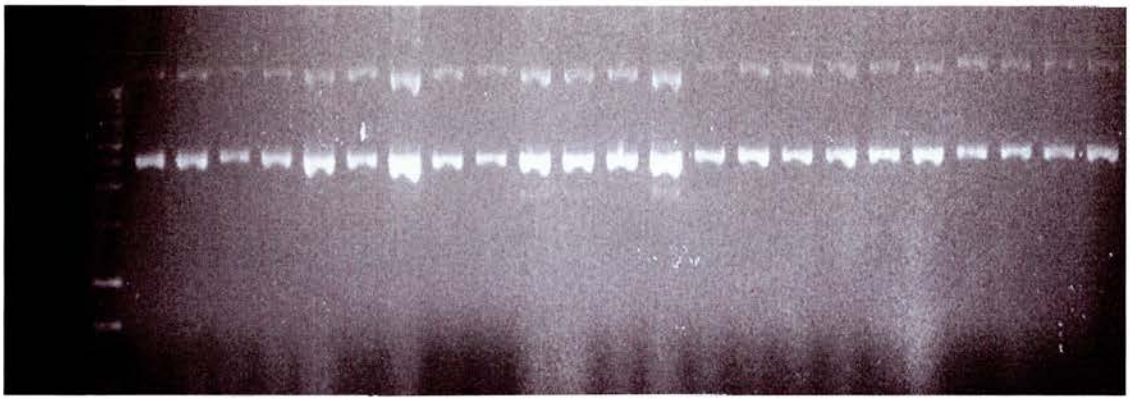


Figure 4-8 Initially unsuccessful ligation products.

Gel electrophoresis of ligation products from both mutant and wild-type constructs showed that these fragments had failed to be incorporated into psp72. A single large fragment should be seen in each lane, instead of which, a separate band of whole psp72 is seen, implying failure of the original digest.

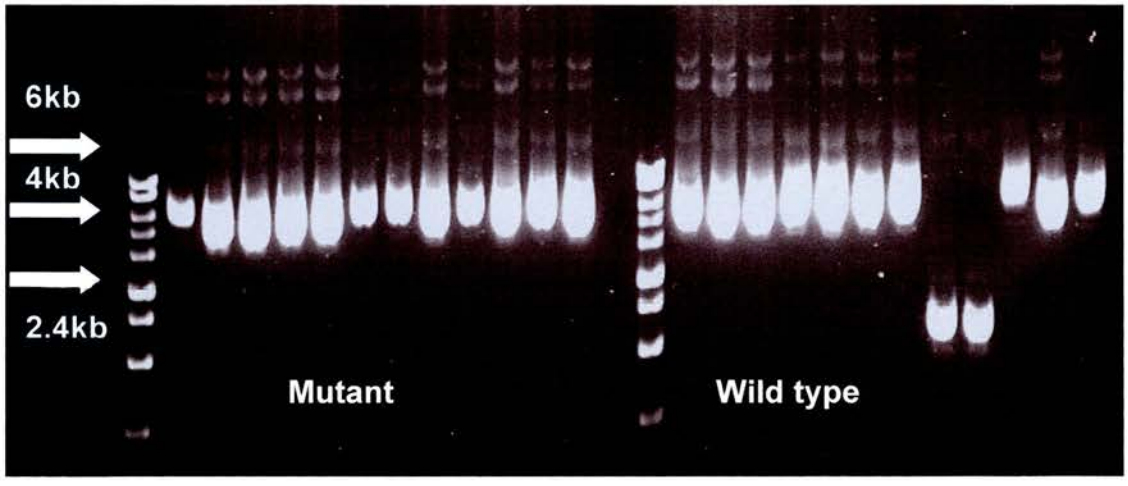


Figure 4-9 Successful ligation of PPARγ into pSP72 vector

Successful ligation of PPARγ into pSP72 enabled bacterial transformation to produce large masses of this intermediate construct. Suitable colonies were picked and DNA from plasmid mini-preparations electrophoresed on agarose gels to assess whether the PPARγ sequences had inserted correctly. A fragment size of approx 4 kb corresponded to the correct insertion. Smaller fragments seen correspond to vector alone with no incorporated DNA.

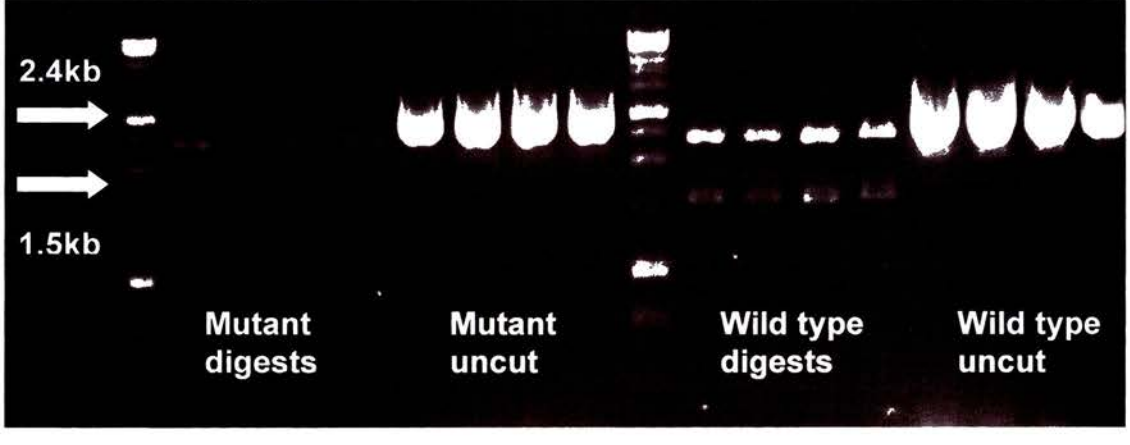


Figure 4-10 Restriction digests of pSP72/PPARγ constructs showing correct insertion of wild type and mutant PPARγ sequences.

Digestion of the PPARγ/pSP72 construct with XhoI/XbaI shows two fragments of 1.5kb and 2.4 kb respectively, demonstrating that the PPARγ fragment had correctly incorporated into the pSP72 vector.

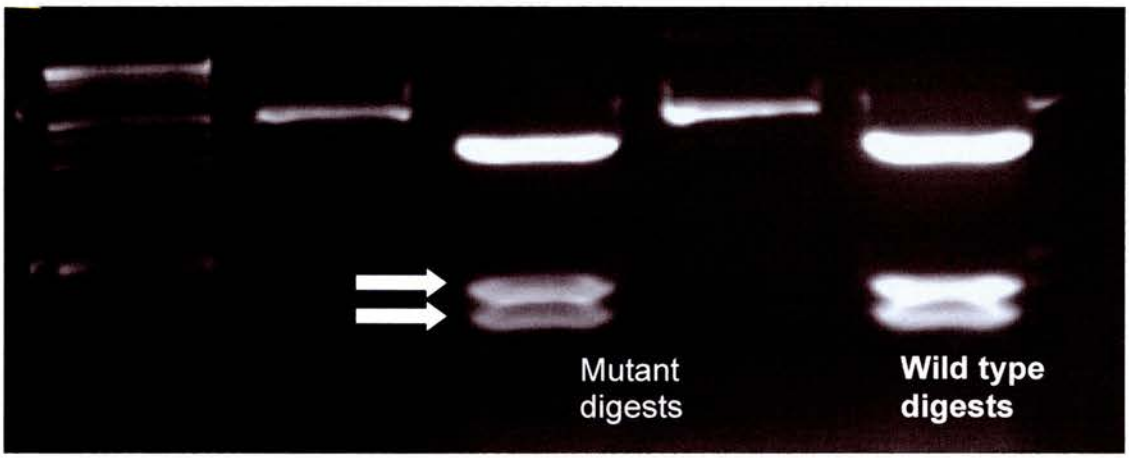


Figure 4-11 Restriction digests of pSP72-PPARγ with EcoRI/XhoI.

An attempt to excise PPARγ from pSP72 and subsequent insertion into pIRES-GFP was made using XhoI and EcoRI. This resulted in extra fragments being produced (marked in lane 3 and also seen in lane 5) as the full details of restriction digest sites within the PPARγ sequence itself were not known. Unfortunately this meant we could not use this strategy, and had to resort to the use of XhoI and SmaI. (1kb ladder in lane1; uncut mutant and wildtype pSP72/PPARγ lanes 2 and 4).

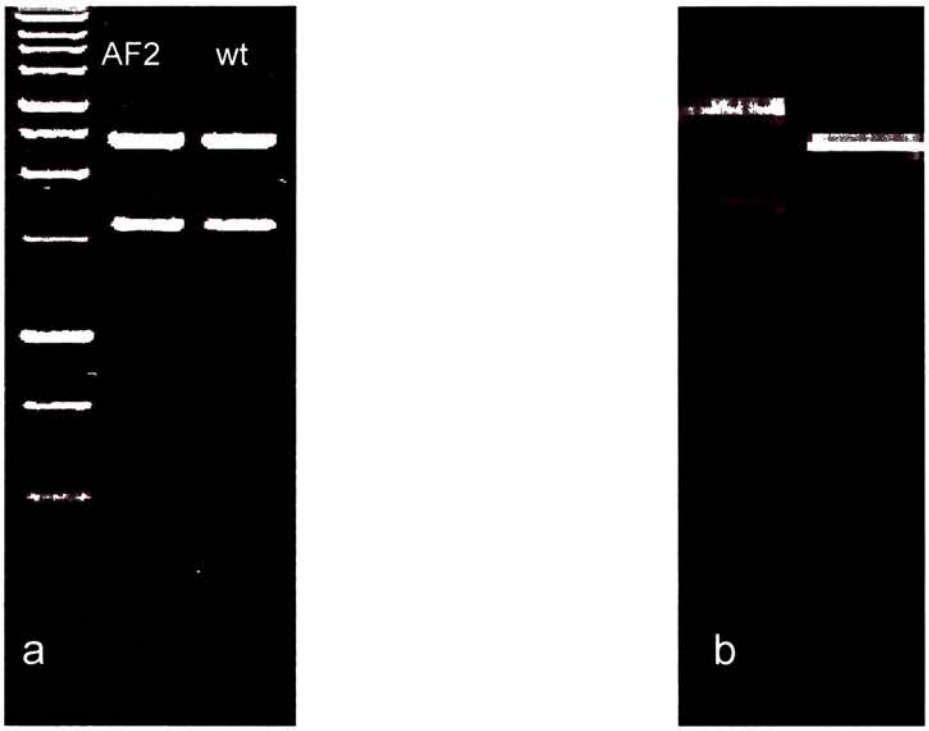


Figure 4-12 Restriction digests of pSP72/PPARγ construct and pIRES2

pSP72/PPARγ (AF2 and wild type) were cut with XhoI and SmaI (Figure 4.12a), revealing a 2.4kb vector fragment and a 1.5kb PPARγ insert bearing complementary ends for ligation into pIRES2-GFP.
pIRES2 –GFP was also cut with SmaI and XhoI (Figure 4.12b). This digest produced a small 40bp fragment between restriction sites, which was not visible on the gel shown. The large residual vector fragment (just under 5.3kb), bearing complementary ends for ligation was clearly visualised and further purified by gel extraction prior to ligation with the PPARγ fragment.

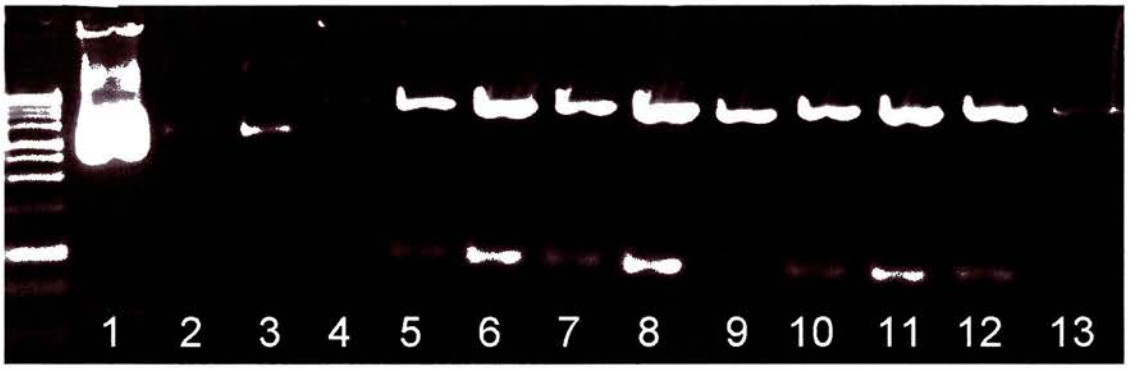


Figure 4-13 Restriction digests of pIRES2/PPARγ ligation products.

DNA mini-preparations from organisms transformed with pIRES2/PPARγ ligation products were digested with BamHI, to assess whether PPARγ had been inserted into the pIRES vector correctly.

Lane 1 is a 1kb ladder.
Lanes 2-4 show uncut pIRES, pSP72/PPARγ mutant, and pSP72/PPARγ wildtype after digestion with BamHI. The small 500bp fragment for the latter two digests is not clear here.
Lanes 5-8 show pIRES2-GFP/PPARγ wildtype digested by BamHI. A large 6kb and small 1 kb fragment are seen as predicted in lanes 6-9.
Lanes 9-14 are pIRES2-GFP/PPARγ mutant, digested with BamHI. Lanes 11-13 have correct digestion products, confirming correct orientation of the insert.
To finally cross-check this data, further BamHI digests were performed on all vectors used in this cloning strategy.

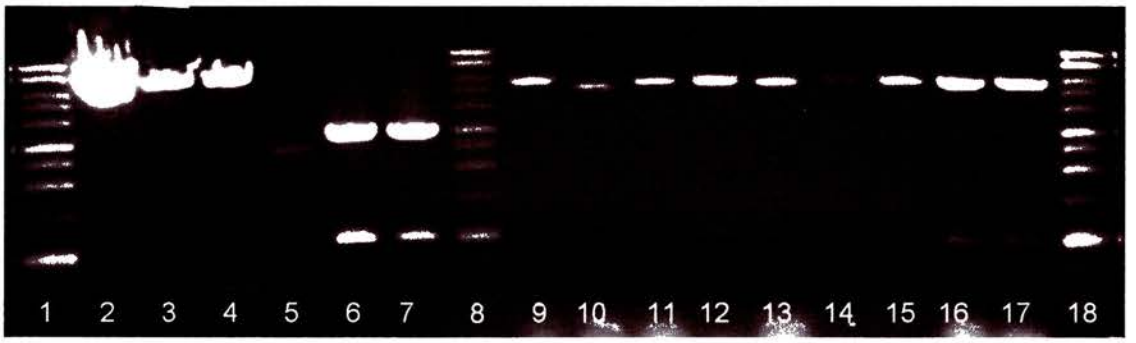


Figure 4-14 Confirmatory BamHI restriction digests on all vectors

Lanes 1, 8, 18 are 1kb ladders.

Lanes 2-7 are: empty pIRES2 (partial linearization); pcDNA3/PPARγ mutant (6.5kb+0.5kb fragments); pcDNA3/PPARγ wild-type (6.5kb+0.5kb fragments); pSP72 (linearised vector); pSP72/PPARγ mutant (3kb+1kb fragment); pSP72/PPARγ wild-type (3kb+1kb fragment).

Lanes 9-13 are pIRES2-GFP/PPARγ mutant ligation products. All but lane 10 show a 6kb+1kb fragment displaying correct ligation of PPARγ into pIRES2.
Lanes 14-17 are pIRES2-GFP/PPARγ wild-type ligation products. All except lane 14 show the same 2 fragments. DNA from clones with correct digestion products were used for transfections.

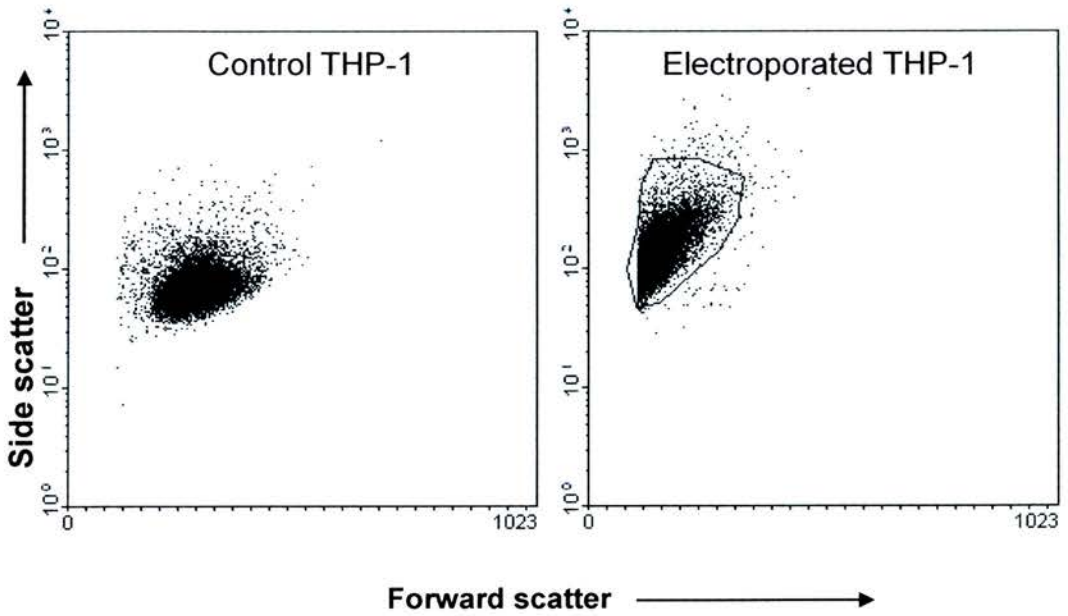


Figure 4-15 THP-1 scatter shift after electroporation

Using both EquiBio and Amaxa electroporation pulse field generators, THP-1 cells were exposed to electric pulses of varying strength and duration. Electroporation altered forward and side scatter (see also Figures 4-17-4-20). Trypan blue staining showed a 95% total death rate in electroporated cells, deemed acceptable for these protocols. Trypan blue exclusion was maintained at 48 hours for all the profiled THP-1 cells.

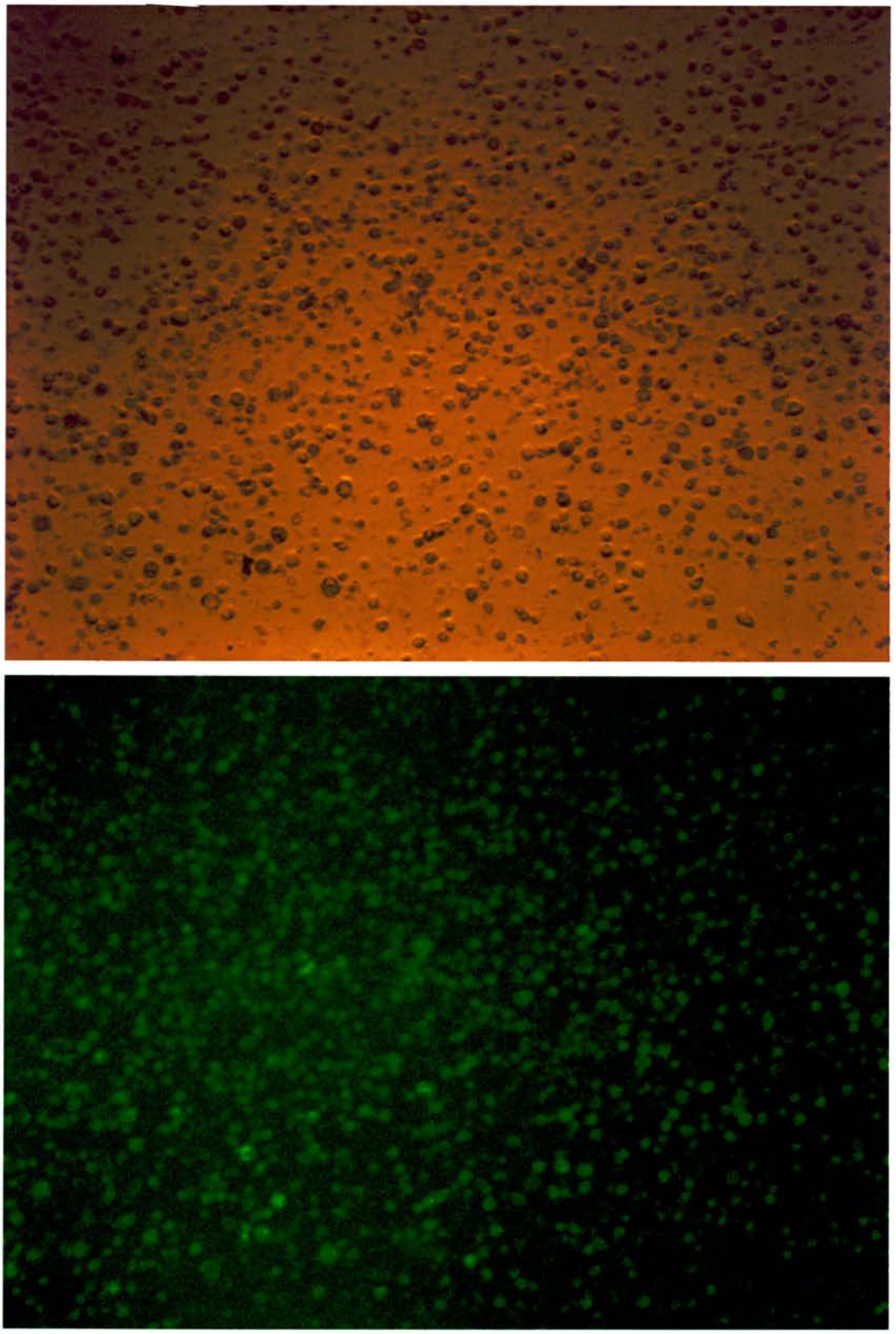


Figure 4-16 THP-1 cell microscopy following transfection

THP-1 cells examined under white light (above) and green fluorescent light (below) following transfection

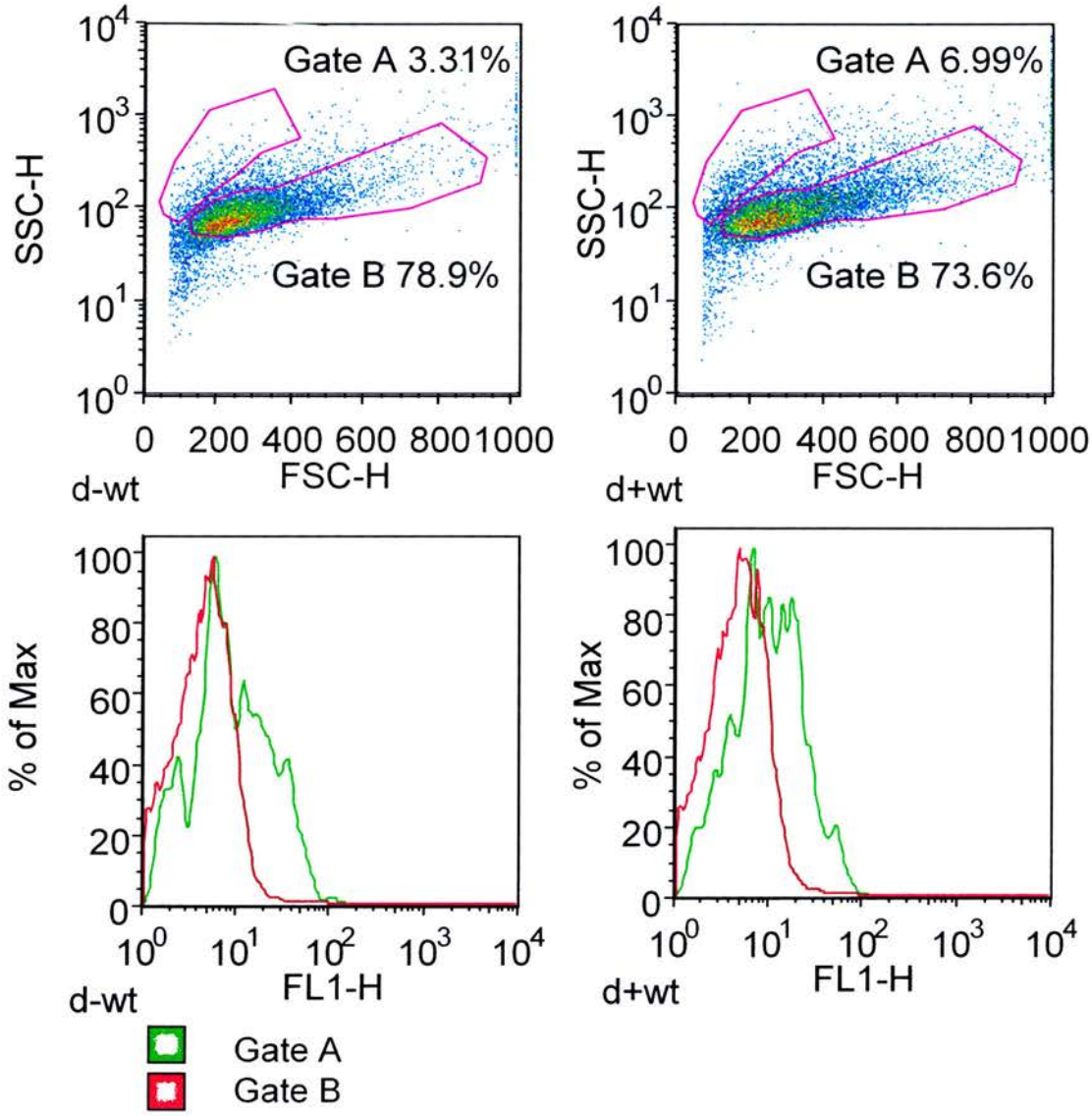


Figure 4-17 Double pulse wild-type PPARγ transfection

THP-1 cells were electroporated with a double waveform electric pulse, with (right hand panels) and without (left hand panels) the pIRES/PPARγ wild-type construct. All electroporated cell scatter profiles, with or without vector/construct DNA, showed a higher side-scatter sub-population as in Figure 4.1.5. Gating on individual populations permitted FL-1 fluorescence to be quantified. Cells were allowed to recover for 48 hrs following electroporation before flow assessment was performed.

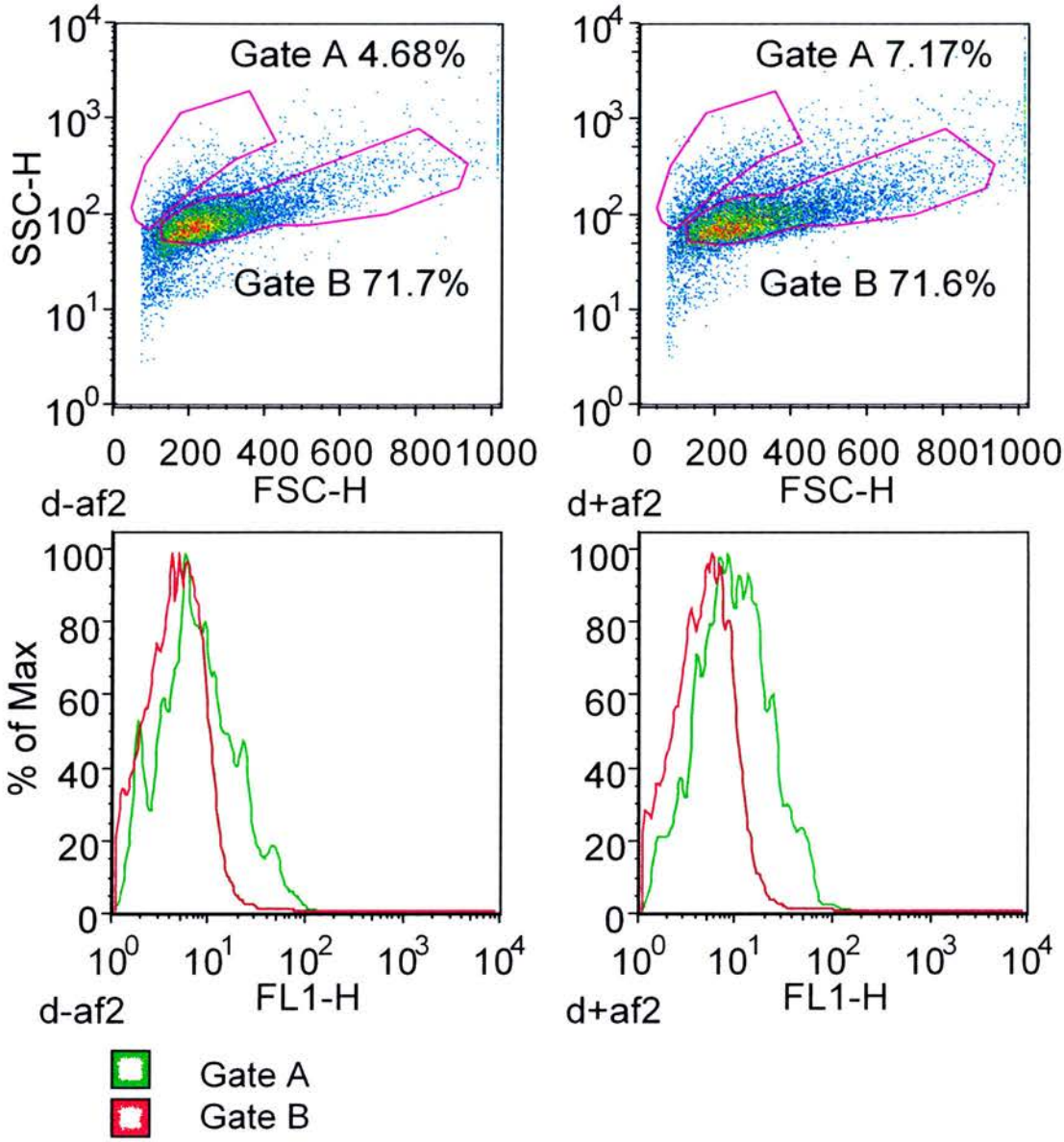


Figure 4-18 Double pulse mutant PPARγ transfection

THP-1 cells were electroporated with a double waveform electric pulse, with (right hand panels) and without (left hand panels) the pIRES/PPARγ mutant construct (termed af2 in figures).
Little change in cell scatter profile in comparison to wild-type transfection was seen. No difference in fluorescence in the higher side scatter population was seen.

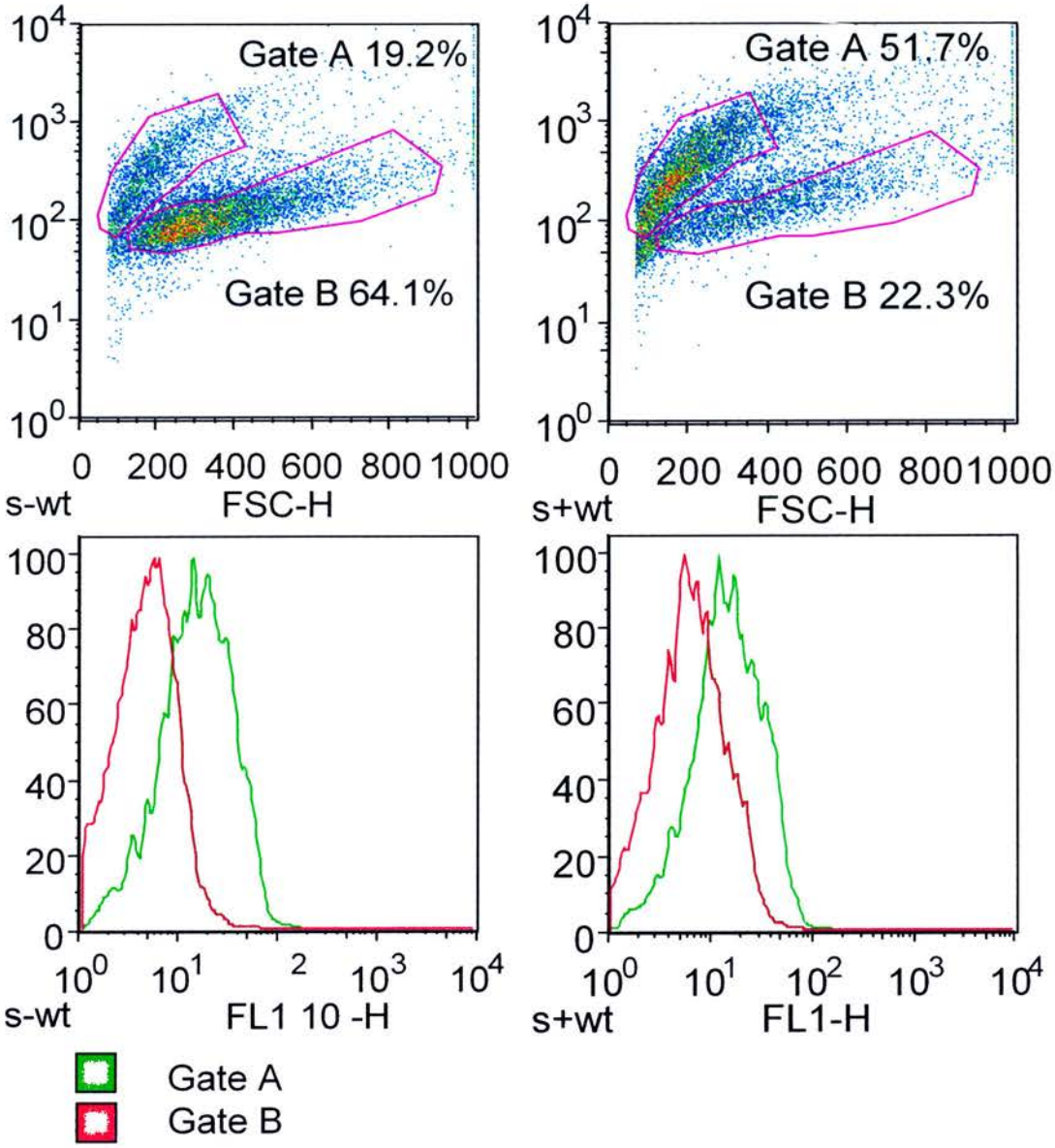


Figure 4-19 Single pulse wild-type PPARγ transfection

THP-1 cells were electroporated with a single waveform electric pulse, with (right hand panels) and without (left hand panels) the wild-type PPARγ construct.
A noticeable increase in cell scatter profile in cells electroporated with vector/construct DNA is seen in comparison to sham transfections with buffer alone. However, although the higher side scatter populations show higher FL-1 fluorescence, this is the same for both sham and vector/construct electroporations.

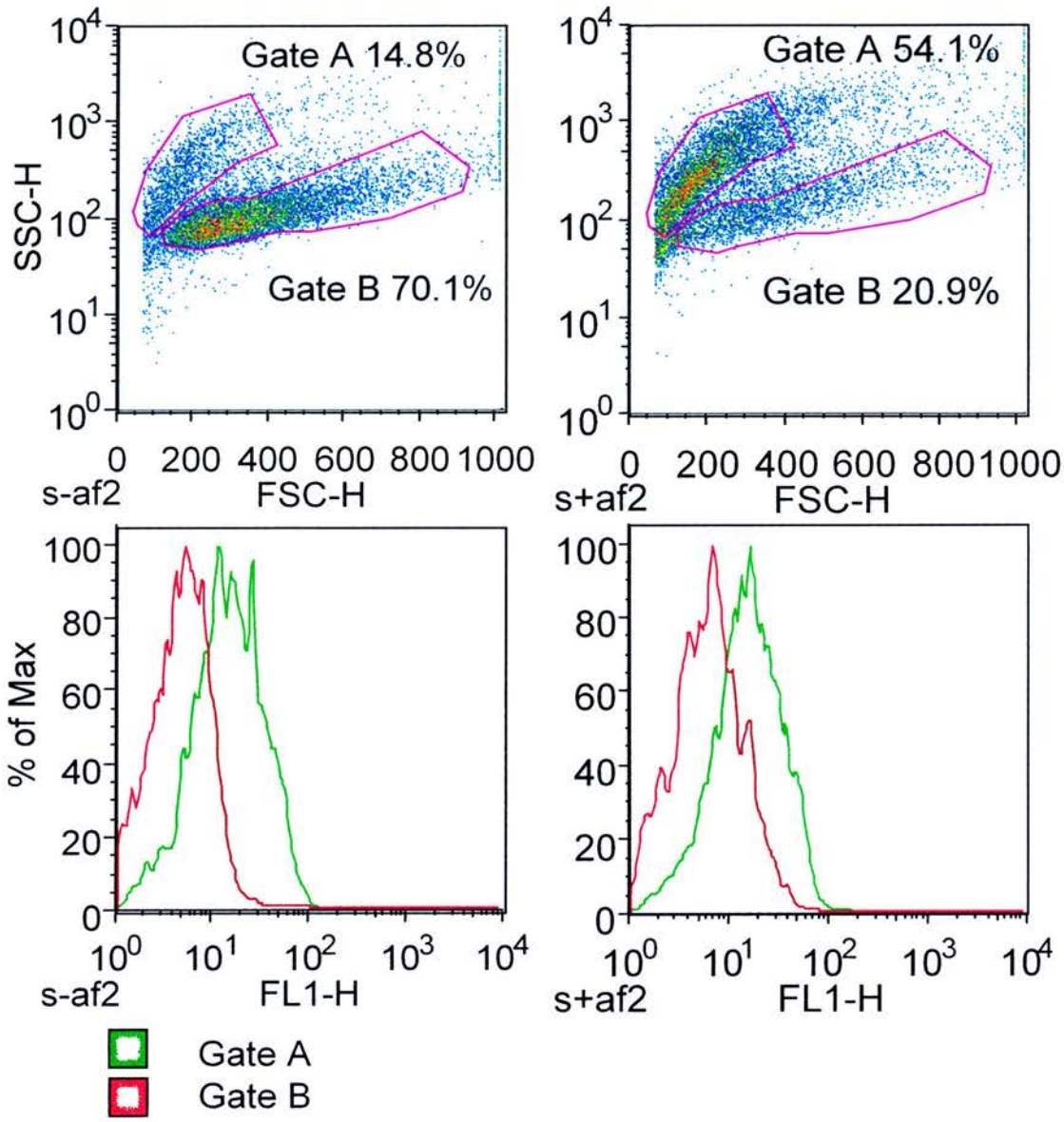


Figure 4-20 Single pulse mutant PPARγ transfection

THP-1 cells were electroporated with a single waveform electric pulse, with (right hand panels) and without (left hand panels) the pIRES/PPARγ mutant construct (termed af2 in figures).
A similar increase in cell scatter profile in cells electroporated with vector/construct DNA is seen in comparison wild-type transfections. However, no noticeable difference in FL-1 fluorescence is seen between sham and vector/construct electroporations.

Chapter 5 TRANSCRIPTIONAL PROFILING OF MONOCYTE RESPONSES TO MICROENVIRONMENTAL CHANGE

5.1 Introduction: assessing gene function

Definitions of lipid-laden cells within atherosclerotic plaque have been problematic. The monocytic origin of lipid-laden foam cells seen in most areas of atherosclerotic plaque has only been established in the last two decades. Cellular changes at a protein level are governed in part by transcriptional responses (Brenner, Jacob, & Meselson 1961; Crick et al. 1961). A high throughput screening strategy was therefore sought to efficiently examine early gene responses to modified lipoproteins.

5.1.1 Expression profiling: assessing cell responses at a gene level

An alternative route to examine the roles of an individual gene in cellular processes is to determine expression patterns. Gene expression profiling allows analysis of gene activity in different tissues, similar tissues at different stages of development, or contemporaneously comparing cell responses following stimulation with different stimuli. High throughput screening technologies such as micro and macro arrays, allow the multiple analysis of several hundred or several thousand genes at one time, with obvious applicability for studies relating to drug development, embryology, cellular and molecular pathology and microbiology.

Many traditional assays of transcription may be pursued based on the principle of hybridization between nucleic acid pairs, one of which is immobilized on a matrix. This is a core technique in molecular biology (Southern, Mir, & Shchepinov 1999) offering highly sensitive and specific detection at a gene level by exploiting the natural, mandatory and exclusive complementary binding between nucleic acids strands (Gillespie & Spiegelman 1965). Early applications used purified single labelled oligonucleotide or polynucleotide species in liquid phase with complex polynucleotide mixtures attached to a solid phase. Quantitative information about particular transcripts in pooled RNA could be derived using multiple RNA samples immobilized on the same matrix. mRNA or total RNA was immobilised on membranes, and then incubated with a radiolabelled, gene-specific target. The observation that single-stranded DNA binds strongly to nitrocellulose membranes in a manner that blocked DNA strand re-association but permitted hybridization to complementary RNA formed the original basis of the original DNA 'blotting' methods, combining filter hybridization with gel separation of restriction digests (Southern 1975) and nucleic acid dot-blotting (Kafatos, Jones, & Efstratiadis 1979). Further key techniques,

including the including northern blot analyses (Alwine, Kemp, & Stark 1977), S1 nuclease protection (Berk & Sharp 1977), and differential display (Liang & Pardee 1992), have offered the ability to quantify gene expression. Automation and miniaturisation of the dot-blot proved that hybridisation could be used on a large scale to interrogate cDNA libraries (Adams 1991; Lennon & Lehrach 1991; Okubo 1992). Serial analysis of gene expression (SAGE) has applied expression analysis to small cell sub-populations and micro-anatomic structures, enabling the investigation of small cell or tissue samples (Velculescu et al. 1995). Qualitative and semi-quantitative comparisons of gene transcription may be made using standard reverse transcriptase polymerase chain reaction (RT-PCR) assays (Scharf, Horn, & Erlich 1986), with more robust quantitation available using real-time RT-PCR (Gibson, Heid, & Williams 1996), and RNase protection assays. These labour-intensive methods, although well accepted, can only address the transcriptional profiles of a few genes per assay. Newer techniques now provide the ability to analyse and quantify thousands of messenger RNA (mRNA) transcripts, using gene expression profiling methods (Duggan et al. 1999). Both complementary DNA (cDNA) (Schena et al. 1995) and oligonucleotide arrays (Lockhart 1996) may be used to enable the parallel studies of expression levels in multiple genes. Gene arrays provide information about tissue specificity of gene expression and dynamic information that compares relative expression patterns of genes. Both cDNA and oligonucleotide arrays are capable of analysing patterns of gene expression, but fundamental technical differences exist between the methods. Oligonucleotide arrays involve the bonding of short lengths of nucleic acid, using sequencing information, without the need for PCR reactions or cDNA products. High densities of target genes, in multiples of 104 genes may be spotted onto matrices, usually glass, with high resolution confocal laser scanning used to read bound signals (Lipshutz et al. 1999). A cDNA microarray by contrast involves the use of a non-porous solid supports such as a plastic or nylon membrane, or may also use a glass slide. Up to 10,000 cDNAs can be robotically spotted onto a microscope slide at high-density, facilitating miniaturization and enabling radioisotope or fluorescence-based detection. cDNA sequences are hybridized with a double-labelled probe made from RNA samples, usually by a modification of RT-PCR reactions and the probe labelled either by a radioisotope, or with a fluorescent marker. At the time of commencement of this project, few commercially available arrays were available. Profiling experiments were restricted to facilities with sufficient core expertise in robotics and fluorescence detection to design and manufacture their own arrays, using oligonucleotide-based techniques. In contrast, I sought a commercial array using techniques immediately available to me. A

cDNA array with a relevant selection of genes including those specific to adhesion was a pragmatic compromise between cost and availability.

The first commercially available small-scale gene arrays were based on nylon membrane technology, with gene specific spots of cDNA varying between 200 base pairs and 600 base pairs. A nylon membrane array (Clontech "Atlas" USA) was used, with a range of 588 cDNA sequences specific to known cardiovascular disease processes, enabling a comparison of gene changes to be made in lipid-treated monocytes.

5.1.2 Overview of nylon membrane procedure

Two identical positively charged nylon membranes were used with cDNA from 588 genes of interest bonded to their surface enabling the simultaneous transcriptional comparison of two cellular treatments. Bonded cDNA fragments on the array were originally amplified from mRNA regions lacking repeat elements, high homology sequences or a poly-A tail. Using these cDNA specific primers, and incorporating ^{32}P dATP, it is theoretically possible to detect as few as 20 transcripts per cell. Probe mixtures are synthesised by reverse transcription of 1μg of RNA using a specific cDNA primer mix, tailored to the genes on the membrane: random primer mixtures are not used. The cDNA primer mix contains specific primers for each cDNA sequence on the array. Radioactive labelling is executed by incorporating α-^{32}P dATP into the reverse transcriptase reaction. The labelled probe is then hybridised to the array surface using a hybridisation buffer, similar in composition to that used for northern blots. High stringency washes are performed, following which analysis of signals is carried out after image capture using either autoradiography or phosphor imaging. Probes manufactured in a cDNA-specific way are less complex than those made with random or oligo (dT) primers, resulting in higher sensitivities and lower background signals. Because of this it is possible to use probes manufactured from total cell RNA, without the need for poly A+ RNA isolation. This reduces sample preparation time, and the level of RNA degradation per sample. Although poly A+ RNA derived probes are more sensitive than total RNA derived probes, attempts to use this technique, either by classical preparation of poly A+ RNA, or by using poly A+ extraction kits, proved detrimental to RNA quality. Early RNA degradation was seen, and probes produced had poor radioactivity profiles.

Quantification of transcripts is achieved by comparing expression levels of housekeeping genes to target genes (Yang et al. 2002). Alternative strategies for balancing housekeeping gene signals involve either examining signals from individual genes that do not vary in intensity between control and experimental samples, or by taking averages of all housekeeping genes on a membrane, and comparing individual gene signals to a mean for

the housekeeping genes, a technique known as global normalisation (Yang et al. 2002). Use of normalisation techniques offers the ability to quantitatively compare probes manufactured from different cell treatments, possibly with different RNA yields, without concern for artefactually induced quantitative differences. Thus within any one membrane array, actual signals from each individual gene spot are compared to a normalised background signal. Individual gene spots are then compared to signals derived from a comparable second array (Smyth & Speed 2003).

5.2 Specific array methodology and technical data

5.2.1 Preparation of RNA derived cDNA probes for array analysis

Although the responses of mononuclear cells to lipid and PPARγ ligands have been explored, a rigorous definition of early gene changes in a human primary cell *in vitro* system has not been made to date. Monocytes from whole human venous blood were purified by adhesion to tissue culture plastic. Monocytes were exposed to control medium or 50μg/ml ox-LDL for 24 hrs. Cells were then washed and lysed, and RNA extracted from lysates. The stringency for RNA preparation is higher than that necessary for RT-PCR, and this coupled with the limited amount of RNA available from human primary monocytes delayed the isolation procedure.

Preparation of RNA samples used monocytes from MACS preparations with a mean purity of 90% assessed by flow-cytometric scatter plots (see Chapter 6, section 6.2.2). It is appreciated that this means of assessment may underestimate actual purity, because of random scatter events and inclusion of debris, although this is minimised during acquisition. Cation-deplete washes, immunomagnetic depletion using CD61+ve magnetic beads, and monocyte adhesion to tissue culture plastic with further subsequent washes prior to cell treatments all reduced platelet contamination. Initial yields from RNA preparations were low, despite attempts to isolate cells from 240mls of whole blood. Initial attempts to re-precipitate RNA to increase RNA concentrations for probe synthesis were also unsuccessful. Thus for further RNA preparations, at least 240-320 ml were used. Probe synthesis was performed using a modified Reverse Transcriptase (RT) reaction, utilising a commercially supplied mouse moloney leukaemia virus (MMLV) RT enzyme. Radiolabelling was performed with ^{32}P dATP, prepared in a master-mix and added proportionate to the mass of purified RNA used. Specific primers complementary to the cDNA sequences spotted on the array membrane surface were used. An internal control to ensure function of the MMLV RT was performed using a purified Poly A+ RNA sample provided by Clontech. Probe activity was then assessed on a scintillation counter. However after MMLV-RT reactions were

performed, low levels of incorporated radioactivity were seen on elution profiles. Agarose gel electrophoresis from eluted probe showed little cDNA suggesting inefficiency either in probe synthesis or in probe isolation.

Probes were eluted by high-speed centrifugation (15,000*g*, 1min) through Chromaspin™ columns designed to separate unlabelled dATP from incorporated nucleotide within a functional probe. However, only the control Poly A+ RNA succeeded in making a probe with a defined peak of radioactivity, corresponding to specific isotype-labelled nucleotide incorporation (see Figure 5.1). Experimental samples showed suboptimal resolution, without clear peaks of radioactivity, and thus by definition no selective incorporation of ^{32}P dATP had occurred, indicating unsuccessful probe synthesis. In particular, the expected differential activity from unincorporated free ^{32}P dATP isotope was not separate from the anticipated active probe. To address this, an alternative mode of probe separation was sought. Alternative micro-centrifugation columns were sourced, similar in composition to Sephadex G50 columns used for nucleic acid or protein separation. Active probe was initially bound to these "Nucleospin" columns. After serially washing the column an elution buffer was added and the whole fraction of probe eluate collected. This method removes unincorporated nucleotide, by serial washes, and allows recovery of whole incorporated probe. Activity of this single fraction of probe was quantified using scintillation counts running on a specific ^{32}P programme. Initial attempts to manufacture probe were thus severely limited by probe isolation techniques. Initial sample sizes were of the order of 50ng per µl, and purity by gel electrophoresis was high. Despite this low levels of incorporated probe were found. A repeat monocyte RNA preparation from 320 mls whole blood showed clean RNA separation, with no gel electrophoresis evidence of RNA degradation. Control RNA had a total mass of 32.5µg and ox-LDL treated samples a mass of 34µg following DNase treatment. A260/280 absorbance ratios were 1.97 and 1.92 respectively, confirming high RNA purity.

Probe synthesis was performed according to the commercial recommendations, and probe separation carried out by elution using nucleospin columns, from Clontech (see Chapter 2).

Internal control blank membranes were also exposed to active probe to ensure no significant background signal was present. These membranes were hybridised and exposed at the same time to ensure consistency of hybridisation and exposure conditions. Once elution of clean probe was achieved, hybridisation was performed with excellent radiographic results using both autoradiography, and storm phosphor imager analysis. After processing had been terminated and image acquisition completed, unquantified phosphor images were available, (Figure 5.2).

5.2.2 Data analysis

For data analysis of gel images, two strategies were used. Firstly, using Molecular Dynamics ImageQuant software, background signals were assessed both across the whole membrane and also locally around each signal spot. This was subtracted from high-level signals giving an assessment of net signal strength. Secondly, housekeeping genes were assessed for their expression, and averages of these were taken, either by manual point measurements or by linear averages through a line of gene spots. This was then used to calculate a ratio for each membrane, giving a figure in proportion to housekeeping gene expression. Finally the net signal ratio to control for each gene was then calculated to compare control with experimental samples. Gene expression changes using this method are catalogued in the Chapter 5 data tables in the attached Appendix.

The second strategy was to use commercially available software (Atlas software, Clontech, USA), which automatically adjusts for discrepancies within individual membranes. This technique allows for the discarding of poor quality signals that may confound analysis e.g. by preventing a "bleeding" spot to confound neighbouring signals. Corroboration of data between the two methods was sought. Results were expressed as a ratio to normal. Genes were stratified according to the change they showed either below or above baseline expression. Genes with transcription changes at a 2-fold or higher level are immediately obvious using a colour-coded readout, (Fig 5.3).

5.3 Results

5.3.1 Trends in gene expression changes

Assessment of data from the gene array confirms certain documented transcriptional changes occurring following macrophage exposure to lipoproteins, and particularly to ox-LDL. These include the up-regulation of orphan nuclear receptors (Nagy et al. 1998), LDL receptor (Fogelman et al. 1988), and LDL-related protein (Watanabe et al. 1994).

It was useful to examine expression changes in clusters of functionally related genes, and full data tables of phosphor-imaging signal strength are presented as supplementary data tables, (Chapter 5 Appendix). Raw data processing suggested that multiple genes governing lipid metabolism, matrix adhesion and platelet adhesion were up-regulated by at least 2-fold following ox-LDL exposure, including genes encoding integrin αv, α3, β2, β3, β5, and β7 subunits. Lecithin-cholesterol transferase expression was increased, and up-regulation of genes for multiple collagen types and collagen precursors were apparent, as well as changes in integrin signals for ICAM-2, PECAM and P-selectin. Increases in gene expression for angiopoietin-1 and plasminogen activator inhibitor-3, with a reduction in tissue plasminogen

activator, suggested altered vascular growth influences and effects upon local fibrinolysis. Other changes with potential local pro-thrombotic effects incuded increased expression of thrombin receptor, anti-thrombin III, and platelet-activating factor. A large number of cytochrome P450 genes were upregulated, suggesting the possibility of alterations in cyclic GMP flux, and increased oxidative capacity of ox-LDL exposed macrophages. However, use of the Atlas software package did not confirm all of these gene changes. For this reason, only genes showing high levels of altered transcription using both software analysis methods were focused upon.

To examine marked gene expression changes, only genes demonstrating 3-fold changes using both software techniques were looked at. Target genes deemed of interest and seen to change by this amount were CD47 and LFA-1α subunit. A 2-fold decrement in tissue plasminogen activator (tPA) was noted. Although this was a less pronounced change, tPA was deemed to be a relevant target in the context of atherothrombosis, and was further investigated.

5.3.2 Confirmation of gene array results by RT-PCR

Mini-arrays offer a discrete amount of profiling data in a more tailored format than larger-scale array technologies. Nylon membrane arrays thus offer a reasonable first screen to assess transcriptional changes. However, resources limited repetition of each array, and individual gene responses were thus further investigated by RT-PCR. Primers were designed for CD47 and LFA-1α subunit and tPA. A panel of primers was used with varying melting temperatures, positions and product lengths, (Chapter 2 section 2.10.8). Samples of RNA from MACS-purified monocytes cultured for 24 hours in ox-LDL or control media were used for RT-PCR. Despite the use of a large number of computer-designed primers, no satisfactory PCR product was obtained for tPA analysis. Each polymerase chain reaction was optimised by titration of temperature and magnesium ion concentration. Annealing temperatures were calibrated to primer length and base-pair composition. Samples of RNA used in these reactions were run contemporaneously with other templates successfully, proving high quality control. Despite these troubleshooting strategies no successful PCR reactions occurred with tPA, precluding confirmation of altered levels of expression. No problems were encountered for the other two target genes. PCR over 35 cycles showed a down regulation in LFA-1α (Figure 5.4). In monocytes matured to 5 days in ox-LDL the levels of LFA-1α transcription appeared to recover, suggesting a transient down-regulation only on initial exposure to ox-LDL (Figure 5.4). Down-regulation in CD47 was less clear (Figure 5.5). Although the GAPDH control suggests the ratio of GAPDH to CD47 has

increased, it was not as unequivocal as with LFA-1α. Although the relative ratio of CD47 to GAPDH appeared slightly lower in ox-LDL treated cells, this was not as clear as on the array. Moreover, any differences between control and ox-LDL treated samples were no longer evident in RNA prepared from 5-day old macrophages. This transient change in transcription suggests that, as with the changes seen in LFA-1α transcription, there may be compensatory changes made with increasing maturation.

5.3.3 Protein level corroboration of array data

The phenomenon of a transient reduction in gene transcription that subsequently returns to baseline may be due to cells in culture recovering from the effects of lipid exposure. Furthermore, transient changes in transcription may not be ultimately reflected in functional cellular changes as protein expression may remain stable. For this reason, despite repeating the PCR data on 3 different donors, CD47 and LFA-1 cell surface protein expression was assessed by flow cytometry. Monocytes isolated by MACS were cultured in suspension or on plastic, with supplements of ox-LDL or control medium. Cells were phenotyped with un-conjugated primary anti-CD47 (clone B6H12) or anti-LFA-1α (clone Wac70) monoclonal antibodies and a secondary goat-anti mouse antibody conjugated to FITC. No alteration in LFA-1α levels in suspension-cultured monocytes was observed following Ox-LDL exposure (Figure 5.6). There was an increase in mean auto-fluorescence as noted for other phenotyping data in lipid-cultured cells. Cell viability was maintained throughout. Cell population purity was maintained as denoted by scatter plots and also gating over CD14 positive samples.

Flow-cytometric data on CD47 showed parity at all stages of cell maturation in LDL supplementation between ox-LDL, native LDL and control media (Figure 5.6). Cells matured up to 5 days in suspension showed no reduction in CD47 signal. To reproduce adhesion conditions used during RNA preparation for array analysis, monocytes were matured on plastic in lipid-supplemented media for 5 days. After being harvested by trypsin/EDTA treatment, they were labelled with B6H12 and flow cytometrically assessed. No reduction in signal was noted.

5.3.4 Further corroboration of LFA-1α data

A time-course of LFA-1α expression was performed to see if a lag phase existed during which protein was lost from the cell surface. Samples cultured in suspension showed clear expression of LFA-1α at 3 and 5 days. Monocytes matured in ox-LDL adherent to tissue culture plastic, (reproducing the purification procedure used for RNA preparation from

monocytes), showed a discernible reduction in LFA-1α signal (Figure 5.7). A fuller phenotype of monocyte lipid responses was subsequently performed to validate these findings, and is described in detail in Chapter 6.

5.4 Discussion

5.4.1 Gene arrays in the investigation of cardiovascular disease

The use of gene arrays in biomedical investigation has rapidly increased since the period that the work presented in this chapter was performed (Southern 2005). Despite the techniques used in this thesis being now relatively dated, they do offer an overview of the problems inherent in using array-based approaches to explore changes in cellular function and phenotype. Gene profiling in cardiovascular disease is now well established in the fields of atherosclerosis, hypertension and cardiomyocyte research, with many groups adapting gene profiling studies for use in existing animal models of cardiovascular disease (Henriksen & Kotelevtsev 2002).

Cell-culture systems have advantages over animal models in gene micro-array experiments. Using single cell types limits signal dilution or noise due to compared to cell heterogeneity in animal model derived samples (see section 5.5). Tissue culture experiments are comparatively inexpensive offering larger retrievable masses of RNA, allowing flexibility in choosing profiling technologies and platforms. Nevertheless, as tissue-culture systems are by definition remote from human pathology, *in vitro* findings require further validation. After such ratification, often few genes are found to be physiologically relevant to the disease process under study. An example of this was the investigation of hypertension-induced mechanical strain effects on arterial tissue in early atherosclerosis (Feng et al. 1999). Use of a cyclic deformation device on smooth muscle cells in culture allows examination of the cells of interest alone, rather than retrieved arterial sections, and ensures that all cells are exposed to the same stimuli. Despite using a 5,000 gene microarray, only 17 gene changes were noted. Few of these were corroborated by Northern blotting at a transcriptional level, or Western blotting at a protein level.

Similarly small numbers of responding genes were identified in a study designed to identify vascularisation signalling pathways triggered by interactions between factor VIIa and tissue factor in human fibroblasts. Only five up-regulated genes and one downregulated gene were found. Nevertheless, two of the up-regulated genes (Cyr61 and connective tissue growth factor) affect cell adhesion, mitogenesis and migration, relevant to fibroblast proliferative behaviour in the context of intravascular coagulation and vascular injury (Pendurthi et al. 2000).

Time-course expression profiling of "foam-cells" has been attempted using RNA from ox-LDL treated THP-1 cells (Mikita et al. 2001). A previous 10,000-gene cDNA micro-array using THP-1 cells exposed to Ox-LDL showed differential regulation of 268 genes at one time point at least. Integrin encoding genes were noted to be up-regulated including those for α2, α5, αX, β3, β5, and β7 chains (Shiffman et al. 2000). This is of interest, as initial analysis of the nylon membrane array data presented in this chapter suggested up-regulation of αv, α3, β2, β3, β5, and β7 integrins, although these changes were not corroborated by subsequent software analysis. Further phenotypic assessment of monocyte surface integrin expression was thus undertaken using indirect immunolabelling, and is documented in Chapter 6.

Shiffman and Makita also described suppression of cell-cycle genes and changes in both pro- and anti-inflammatory gene transcription, suggesting potentially altered inflammatory function. These observations support previously published work on the anti-inflammatory effects of Ox-LDL blocking I-kB degradation (Hamilton et al. 1998; Page et al. 1999), but no protein level assessment of NF-kB activation was performed. Certainly this would be important given the established pro-inflammatory effects of ox-LDL upon macrophages (Wang et al. 1996). Although findings from these groups are of interest, they focused on the responses of a cell-line with no functional assays performed to explore the impact of transcriptional changes.

5.4.2 Array experimental design and technical issues

The use of RNA from monocytes treated by altered low-density lipoprotein supplements was felt to be crucial to enable an insight into early gene responses to micro-environmental stimuli. Such strategies have been used in other similar explorations of monocyte gene responses, and early work by other groups using large scale gene profiling demonstrated the variable results that individual experiments may produce. In the work shown in this chapter, practical and financial constraints dictated that gene arrays could only be used to "screen" putative candidate genes that might be altered in expression during monocyte exposure to altered lipid environments. Such genes would be explored thereafter using more standardised quantification methods, including RT-PCR, and quantification at a protein level using cell surface immuno-phenotyping. RNA preparation was stringently controlled to prevent RNase contamination, in order to maintain optimal purity for probe synthesis. The methods for this vary according to the assays being performed. It was increasingly apparent while performing this work that higher levels of purity than were acceptable for routine RT-PCR were needed to ensure adequate probe synthesis. Indeed, probe synthesis was critically dependent on

higher levels of RNA purity than those recommended by the manufacturers of the gene arrays (Atlas cDNA Expression Arrays User Manual, Clontech Biotech Corporation USA 1999). Further technical problems were encountered during the probe purification stages. The initial probe elution methods that were suggested by the manufacturer resulted in loss of probe, and indeed there was a continuous washout of probe in the sequential eluates that were produced as part of this method of probe purification. For this reason, an alternative method was used, involving the use of micro-porous membrane centrifugation columns, and an elution buffer, that minimised washing the probe, and maximised probe yield. Whilst this delayed the use of the arrays, it ensured that the probe was of sufficient quality to produce an interpretable high quality signal. Analysis of nylon membrane gene arrays provided another technical hurdle, in the context of quantifying what were essentially radiographs, produced by probe activity. Several control factors were considered. Firstly, background signal levels from control and sample probes and membranes differed visually. Software supplied with both the phosphor imager and subsequent analysis with the gene array manufacturers own software package needed to be used to normalise background levels of signal to avoid misinterpretation. Furthermore, there were issues with excessively high signal levels, producing "bleeds" around particular "hotspots" thus distorting signal interpretation in the area immediately around the gene of interest. Such high-noise areas lead to loss of interpretable signals from adjacent gene spots, with an inability to then compare subtle changes in transcription levels in less highly labelled gene spots. Glass or chip base arrays avoid these issues due to the lower noise levels and more discrete spotting patterns. To assess the contribution that individual hotspots made to background it was suggested that spots should be compared to local area background signal to assess the difference in local signal bias. This was not noticeably different for the majority of spots and thus a general background subtraction was used.

5.4.3 Further assessment of CD47 and LFA-1 changes

A fuller exploration of CD47 and LFA-1 could have been attempted if sub-cellular fractions had been separated, thus offering an opportunity to measure expression within monocyte cell membranes, lipid rafts, and also potentially to look at protein trafficking through organelles.

An active stimulus to remove surface LFA-1α may be necessary to discern a change at the monocyte cell surface. LFA-1α, along with α3β1, and α4β1 integrins, appears not to participate in endocytotic cycling, unlike α5β1, α6β4 and Mac-1 integrins. LFA-1 thus presents a stable profile at the cell surface which may remain constant during monocyte maturation, although low-level cycling of LFA-1 has been reported (Bretscher 1992).

Integrin subunits are integrated within cell membranes, and have strong interactions with structural proteins including talin (Burn, Kupfer, & Singer 1988) and the actin binding protein actinin, the latter shown to specifically associate with β2 integrin chains (Pavalko & LaRoche 1993). Furthermore, discrepancies between transcription and translation are documented for other β2 integrins, notably CD11c/CD18 (Noti & Reinemann 1995), and β2 integrin transcription may be directly regulated during inflammation by hypoxia-inducible factor-1 (HIF-1) (Kong et al. 2004).

Dynamic migration assays might reveal whether changes in integrin transcription correlate with altered leukocyte function in this setting. The use of murine models with gene deletion of LFA-1α (Ding et al. 1999) would be a key strategy to demonstrate an *in vivo* role for LFA-1α in the initiation and progression of atherosclerosis. Biotinylation of monocyte surface LFA-1α would allow integrin trafficking following lipid exposure to be explored in cell lysate sub-fractions, a technique used successfully in assessing α6 integrin mobility (He et al. 2005). This would be of use in assessing any LFA-1 loss during movement through cell matrix components or migration across endothelial monolayers.

5.4.4 Transcriptional redundancy: mismatches between gene signals and protein production and function

Macrophage gene products are under multiple layers of control, with examples including transcriptional and post-transcriptional regulation of M-CSF-1 (Weber et al. 1989). The regulation of IFNγ mediated induction of IL-1 TNFα and uPA is controlled by short lived protein repressors, that act to modulate transcription of these target genes (Collart et al. 1986). Increases in β2 integrin surface expression have been noted to correspond to increased steady state levels of CD18 mRNA in HL60 cells (Hickstein, Back, & Collins 1989). However, it appears that other integrin subunits may undergo post-transcriptional changes, including LFA-1 (Back, Gollahon, & Hickstein 1992). Further control over LFA-1 gene expression operates at a DNA as well as an RNA level, with Methylation of the LFA-1 intron being responsible for altered mRNA levels, a finding that has been matched to higher levels of protein expression (Kaplan et al. 2000). Leukocyte surface molecule expression also depends on intracellular trafficking, and the ability of relevant chaperone proteins to facilitate transport to the cell membrane. Chaperones such as calnexin (Ihara et al. 1999) and calreticulin (Saito et al. 1999) are critical for the delivery of functionally competent proteins to the cell membrane, offering protection from thermal and oxidative stress, and are dependent upon the conformation of protein products. The failure for transcriptional changes

to be reflected at the cell surface reinforces the need to substantiate expression profiling data with further investigation of the relevant proteins themselves.

5.5 Further development of gene profiling

Gene profiling studies demonstrate the potential for new insights into cardiovascular disease processes. Examples include metabolic shift to glucose metabolism during recovery from myocardial infarction (Stanton et al. 2000). Validation of microarray technology to demonstrate biological relevance is currently crucial. Experimental design was deliberately simplistic, using single measurements at single timepoints. It would clearly be better to opt for multiple time points to enable the use of statistical methods for analysis of expression patterns over time or during different cell culture conditions. Moving from *in vitro* work using arrays to sampling from animal models or tissue samples is potentially complicated. Signal dilution of changes in gene expression is a feature of samples containing multiple cell or tissue types. Although laser microdissection techniques are improving the cell-specific isolation of nucleic acids, small sample size, especially in the context of patient biopsies, limits RNA yields. Despite this, studies have been successfully perfomed on aortic arch tissue samples, showing varied EC gene expression due to shear stress (DePaola et al. 1999). Alterations in EC pro-inflammatory and anti-oxidative gene expression have also been highlighted (Passerini et al. 2004). Single-cell or oligocellular PCR-based amplification is thus feasible and allows this technology's use in examining effects at a tissue or organ level.
RNA profiling has aided identification of genetic defects relevant to cardiovascular and metabolic disease (Aitman et al. 1999; Lawn et al. 1999). Nevertheless, RNA expression remains a key focus for most array based experiments, with mammalian genome arrays containing 5,000-10,000 genes now in common use. Current protocols allow reliable detection of messages present at several copies per mammalian cell. Advances in robotics and analysis techniques have meant an improvement in both fabrication and sensitivity. It is now feasible to produce or commercially purchase standardized oligonucleotide and cDNA arrays containing the large sets of human and mouse genes in multiples of 10,000 genes. Careful experimental design is critical to successful utilisation of this technology. Experimental manipulations need to be rigorously controlled. Responses to microenvironment are especially prone to producing misleading expression data. When several thousand genes are being sifted to find variation in small subsets, scrupulous experimental technique is paramount to limit variation. Simple variables including the position of a culture dish in an incubator or the time of day at which an assay is performed, may also be crucial. Comparison of the 'same' experiment performed at different time points

is thus likely to reveal considerably wider variation than seen when the same sample is tested by repeated hybridization.

A significant increase in development and availability of technology has altered the way most laboratories treat the use of gene arrays. From being a specialised tool designed for the wholesale screening of known and unknown genes, arrays have become widely available for repeated transcriptional profiling of multiple cell types under varying conditions (Lobenhofer et al. 2001). The data presented in this chapter is of interest, not merely in terms of the view of cellular transcriptional changes that occur within monocytes in response to altered lipid and inflammatory signals, but also because it provides insight into the design of expression-profiling experiments, highlights the difficulties inherent in such an approach, and offers some lessons in how to refine such experimental strategies. Certainly, with an increase in access to higher throughput profiling tools, any attempt to re-visit this work would involve glass chip arrays. The ability to interrogate multiple sets of gene arrays would clarify those gene targets with greatest transcriptional variation at an earlier stage, possibly allowing for a wider cohort of genes and gene products to be examined by PCR and protein level analyses. Glass arrays benefit from automated signal readout removing operator variability present when using nylon membrane technology. Nevertheless, it is still mandatory to persist with a full investigation of target proteins using appropriate assays.

Information processing will become the greatest challenge for expression profiling experiments (Weinstein et al. 2002). Early studies were deliberately simple, comparing just two samples, with the aim of identifying those genes whose expression levels differed. Increased biological insight is only possible from examining datasets with scores of samples. This requires multiple time points from multiple cell lines treated independently with multiple treatments. Each gene then defines a point in k-dimensional space, where k is the number of samples studied (D'Alimonte et al. 2005). Functional similarities are read as gene clusters in this space. Computational scientists working in array analysis are developing techniques for clustering and anticipating patterns based on established patterns. However, empirical data will still be needed to ratify array findings.

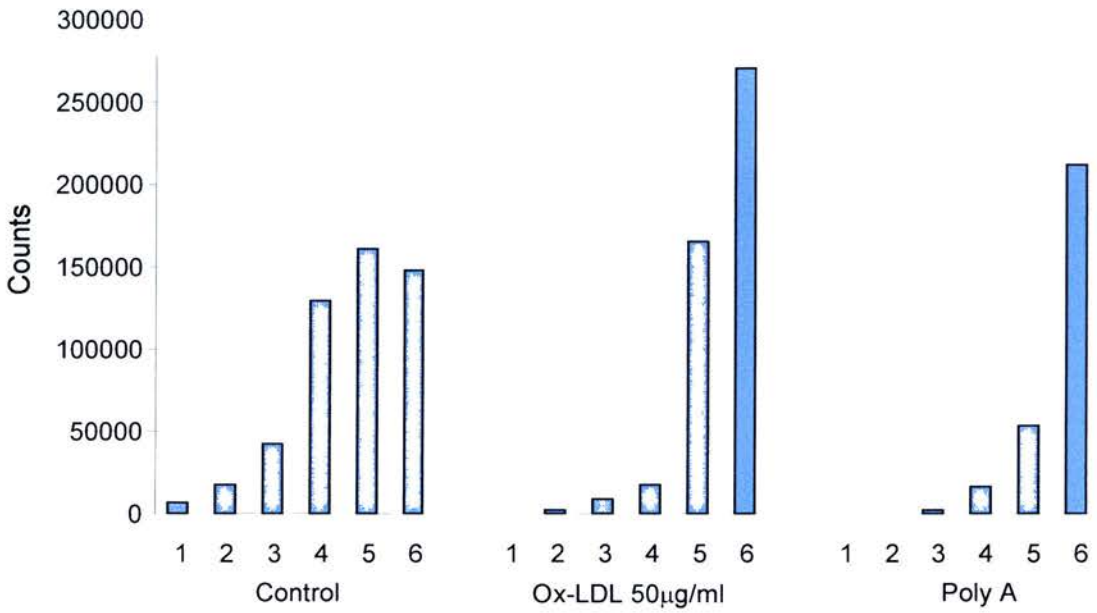


Figure 5-1 Initial probe elution profiles

Initial probe synthesis involved multiple washing steps, resulting in the loss of functional probe. Elution profiles for each probe should display radioactivity peaks. Instead, all samples showed a gradual increase in radioactivity, showing failure of ^{32}P dATP specific. Subsequent probe isolation was performed using micro-porous centrifugation tubes, and subsequent elution with a pH-adjusted buffer. This technique did not require the collection of individual elution fractions. Similar scintillation data is thus unavailable for this technique.

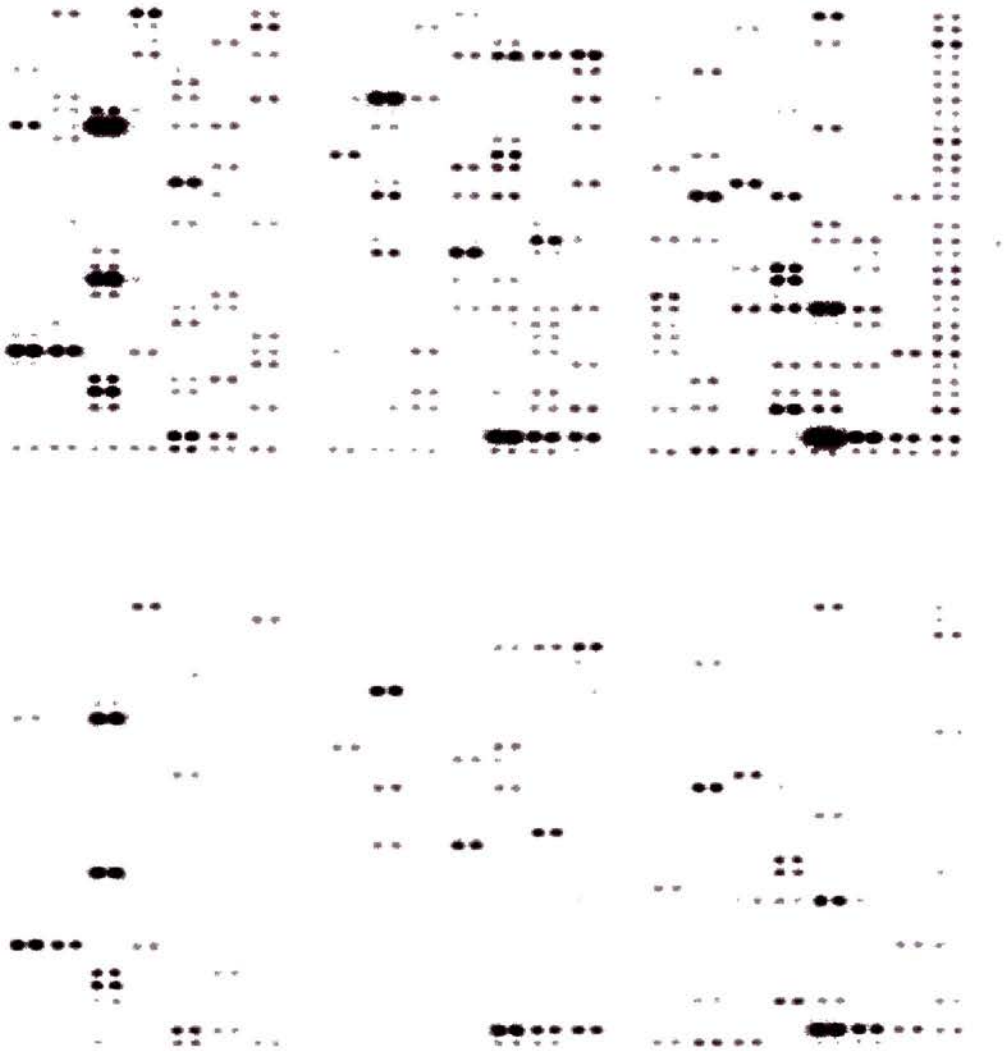


Figure 5-2 Nylon membrane gene arrays derived from RNA of monocytes treated with control (upper panel) and oxidised-LDL (lower panel)

cDNA for individual genes are spotted in 6 grids per array, with house keeping genes in three strips at the base of each array. Differential background signals are compensated for during post-hoc processing.

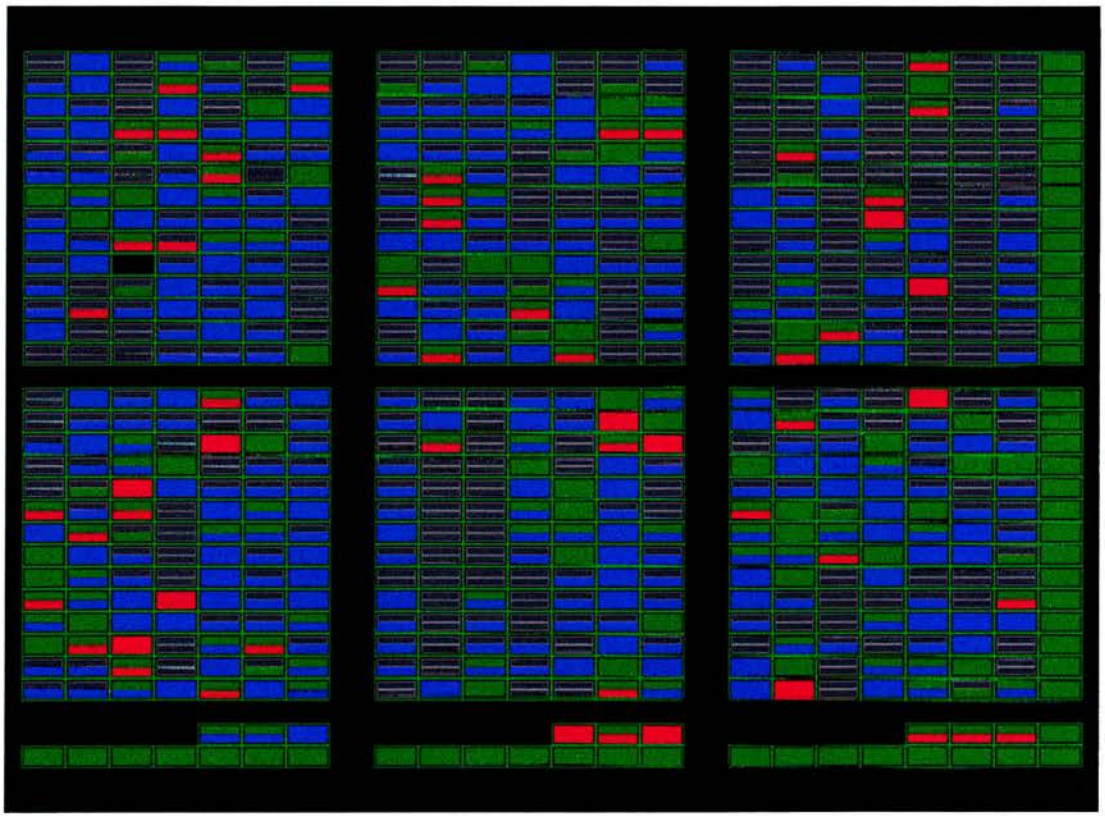


Figure 5-3 Atlas array software analysis of gene expression in control versus ox-LDL labelled probes

Green colour indicates increased expression, red indicates decreased expression. Blue indicates a neutral outcome, i.e. no modulation in gene transcription. Fully filled blocks indicate acceptable signal quality whereas half–filled blocks indicate sub-optimal signal quality. Blank blocks show that no interpretable signal was produced.

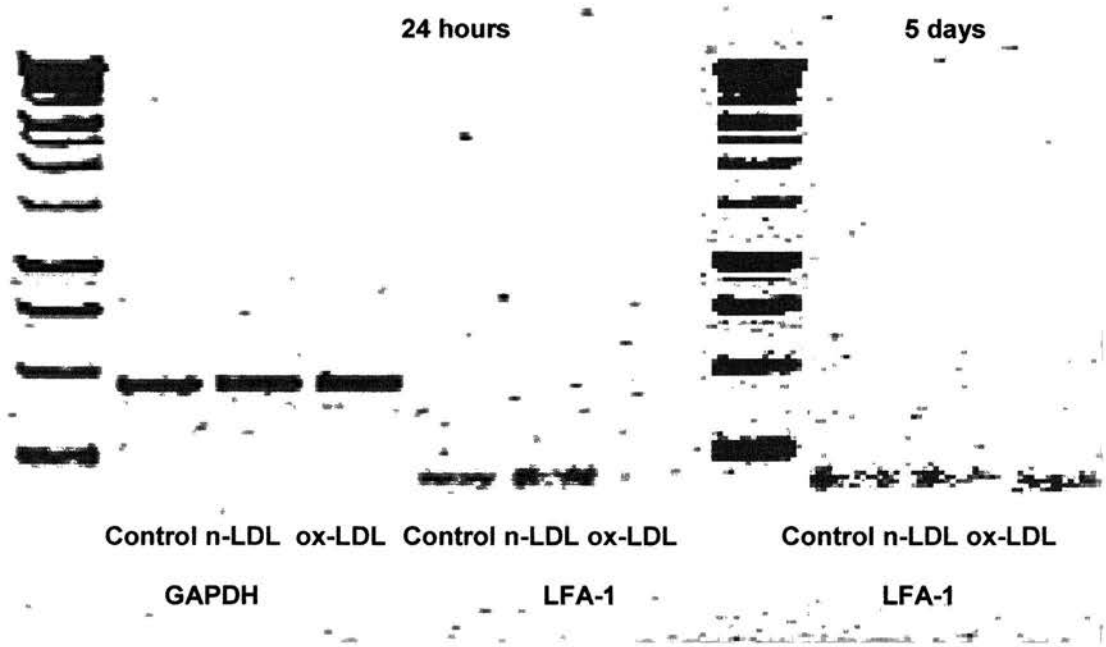


Figure 5-4 Reduced LFA-1α transcription in ox-LDL treated monocytes

RNA from monocytes cultured 24 hours in control media, native LDL or ox-LDL was used in RT-PCR to assay LFA-1α transcription. ox-LDL reduced gene transcription of LFA-1α at 24 hours, with recovery of LFA-1α transcription apparent at 5 days.

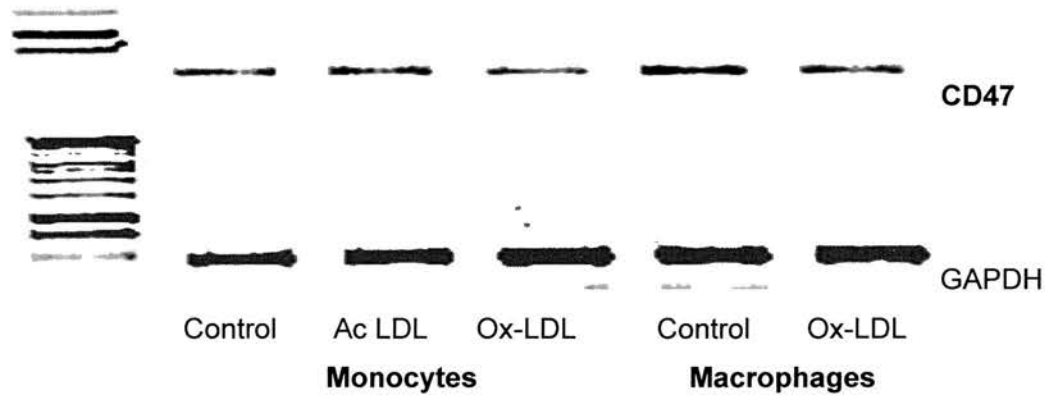


Figure 5-5 RT-PCR of CD47 in naïve monocytes and mature macrophage treated with control media, Acetylated LDL or Oxidised LDL

RNA samples from 24hr old monocytes exposed to control media, acetylated LDL or ox-LDL were used to perform RT-PCR with CD47 primers. Results were compared to GAPDH production for the same RNA samples. 5-day old macrophages were also used to investigate whether the suggested reduction in CD47 transcription was reproduced at later stages of macrophage development.

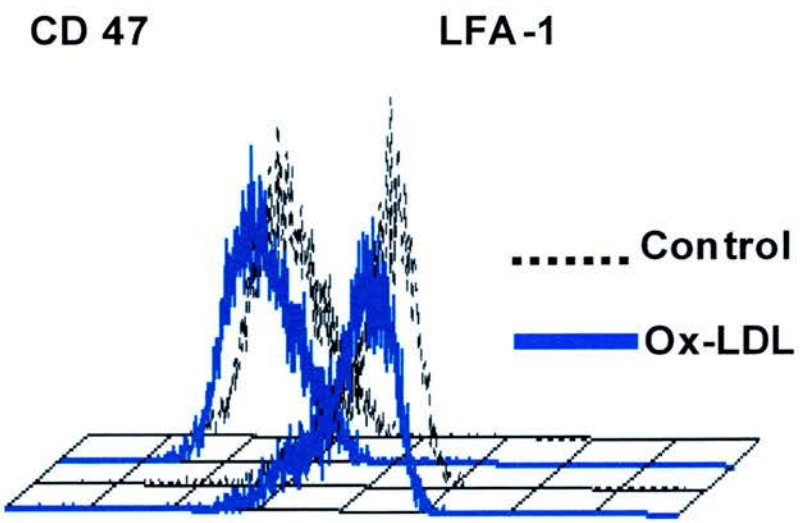


Figure 5-6 CD47 and LFA-1α surface expression is unchanged by ox-LDL

Monocytes cultured for 24hrs in Teflon wells using control media or 50μg/ml ox-LDL supplementation were labelled with monoclonal antibodies against CD47 and LFA-1α. No significant changes in fluorescence levels were demonstrable.

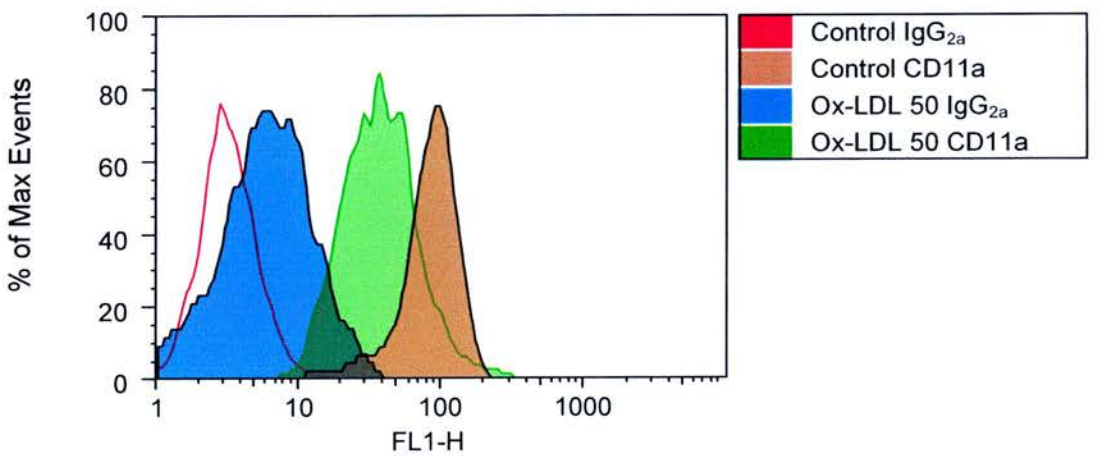


Figure 5-7 Five-day old macrophages display reduced surface expression of LFA-1α after treatment with oxidised LDL

Monocytes matured for five days in vitro on tissue culture plastic either in control media or in media supplemented with oxidised LDL were detached by treatment with trypsin/EDTA 5mM, then washed, immediately labelled and analysed by flow-cytometry. A decrease in LFA-1α surface expression was noted.

Chapter 6 MONOCYTE PHENOTYPIC CHANGES INDUCED BY MICROENVIRONMENTAL STIMULI

6.1 Introduction

Extracellular stimuli alter the structure and function of naïve monocytes, and influence the responses of phenotypically mature resident macrophages at injury sites. Inflammatory cellular responses govern local tissue damage progression, a process highly relevant to vascular injury (Simon et al. 2000b). Naïve monocytes entering active atherosclerotic lesions are exposed to inflammatory stimuli (Plutzky 2001) including altered or modified lipoproteins, locally produced chemokines and cytokines and arachidonic acid metabolites. Pro-atherogenic LDL species may directly modulate vascular adhesion in an oxidation status dependent manner, with vessel wall damage resulting from superoxide mediated injury (Lehr et al. 1992). Ox-LDL contributes directly to endothelial glycocalyx degradation, enhancing leukocyte adhesion and promoting inflammatory vascular wall injury (Constantinescu, Vink, & Spaan 2003). Pro-inflammatory cytokines such as IL-8 reduce production of natural tissue inhibitors of metalloproteinases (TIMPs), (Moreau et al. 1999) promoting local vascular tissue injury. Intra-luminal blood flow may be reduced by subsequent fibroblastic responses during the reparative stages of vessel injury (Lazarous et al. 1996; Schwartz, Majesky, & Murry 1995). Micro-environmental influences upon monocyte phenotype thus govern leukocyte function, and are of direct interest in the study of vascular inflammatory injury. Monocyte surface molecules relevant to atherosclerosis and inflammation were introduced in Chapter 1, but detailed consideration of specific receptors and ligands is warranted. Molecules will be principally referred to by Clusters of Differentiation (CD) nomenclature to keep text succinct, although complex receptors such as integrin heterodimers and Fc receptor subtypes will be referred to by name.

6.1.1 Monocyte pattern recognition receptors in inflammation

In contrast with the large repertoire of rearranged receptors in the acquired immune system, the innate immune system recognises micro-organisms *via* a discrete number of germline-encoded pattern-recognition receptors (PRRs), (reviewed by Akira, Uematsu, & Takeuchi 2006). PRRs are highly conserved through evolution, and have common characteristics, recognising conserved microbial components, known as pathogen-associated molecular patterns (PAMPs). PRRs are expressed constitutively, independent of immunologic memory. Different PRRs react with specific PAMPs, with distinct expression patterns, signalling pathways, and anti-pathogen responses. PRRs include toll-like receptors (TLRs) that detect

bacterial PAMP, scavenger receptors including SR-A and CD36 that may mediate apoptotic cell recognition (Savill et al. 2002) and ingestion of modified LDL (Boullier et al. 2001), and specific receptors for bacterial LPS including CD14 (Landmann et al. 1995). PRR recognition of endogenous ligands, may contribute to chronic inflammatory responses, and are thus of particular interest in conditions such as rheumatoid arthritis and atherosclerosis (reviewed by Karin, Lawrence, & Nizet 2006).

6.1.2 CD14 roles in monocyte differentiation and innate immunity

CD14, a 55kD myeloid membrane glycoprotein linked to the plasma membrane by a phosphatidylinositol phospholipid is present during early and late stages of monocyte differentiation (Simmons et al. 1989). CD14 is important in directing macrophage responses in infection and inflammation. CD14 binds complexes of lipopolysaccharide (LPS) and LPS binding protein (LBP), and is involved in LPS induced TNF-α production and IL-6 and IL-8 release by monocytes and alveolar macrophages (Dentener et al. 1993). CD14 expression is upregulated by LPS and downregulated by IFN-γ and IL-4 (Landmann et al. 1991). Apoptotic cells interact with a region of CD14 closely associated with a known LPS binding region, triggering apoptotic cell phagocytosis but, unlike LPS, without provoking pro-inflammatory cytokine release (Devitt et al. 1998). CD14 ligation enhances LFA-1/ICAM-1 interactions *via* protein kinase mediated coupling, involving CD14, Rho, and PI 3-kinase (Lauener, Geha, & Vercelli 1990). This converts low avidity LFA-1 into an active form capable of increased binding to ICAM-1 (Hmama et al. 1999), enhancing monocyte adhesion.

CD14 expression is an indicator of monocyte viability, purity and normal maturation in culture (Ziegler-Heitbrock & Ulevitch 1993). Monocyte surface CD14 is shed during apoptosis, a mechanism that may be driven by the action of a cell surface phospholipase (Heidenreich et al. 1997). Reduced monocyte CD14 expression may thus suggest *in situ* cell death (Heidenreich 1999), but may also be a function of extrinsic IL-4 stimulation (Lauener et al. 1990), or further differentiation away from a monocyte phenotype, e.g. following vitamin D_3 exposure (Zhang et al. 1994). Cholesterol may be critical in maintaining expression of GPI anchored membrane proteins, and it has been shown that lipid depletion *in vitro* reduces monocyte CD14 (Esfahani et al. 1993). To date, no assessments of surface CD14 during exposure to native or modified low-density lipoproteins have been published.

CD14 binding to LPS primes granulocytes for leukotriene B4 (LTB4) synthesis, in a plasma-dependent manner (Surette et al. 1993; Surette et al. 1998). LPS also primes monocytes to release and metabolise arachidonic acid in response to fMLP. LPS priming of monocytes at

very low concentrations (as low as 10^{-6}mg/ml) is dependent on plasma and CD14 (Surette et al. 1996). Although direct LPS stimulation was not the focus of interest in these experiments, the possibility that CD14 might be modulated by cyclopentenone arachidonic acid metabolites was intriguing. This hypothesis, if true, might reveal a potential regulatory mechanism for CD14 expression.

6.1.3 Role of the scavenger receptor CD36 in monocyte

The leukocyte differentiation antigen CD36, also known as platelet glycoprotein IV (GP IIIb), is a receptor for thrombospondins a group of widely distributed multimodular secreted proteins associated with the extracellular matrix (Bornstein 2001) and possessing potent angiogenic activity (de Fraipont et al. 2000). CD36 is multi-functional. Present on erythrocytes, (Van Schravendijk et al. 1992) it governs the cytoadherence of erythrocytes parasitised by *Plasmodium falciparum* (Oquendo et al. 1989). Platelet surface CD36 is the primary receptor for platelet-collagen adhesion (Tandon, Kralisz, & Jamieson 1989) and partially mediates platelet-monocyte binding (Silverstein, Asch, & Nachman 1989). In monocyte/macrophages, CD36 mediates binding to type I collagen (Janabi et al. 2001) and is implicated in phagocytic macrophage binding to apoptotic granulocytes, utilising thrombospondin as a molecular bridge with the αv integrin chain/vitronectin receptor CD51 (Savill et al. 1992). Recent data show that CD36 plays an anti-microbial role in the recognition of *Staphylococcal aureus via* toll-like receptors 2 and 6 (TLR-2/6) (Hoebe et al. 2005).

Carotid atheroma from hyperglycaemic patients shows elevated expression of CD36. Human macrophages differentiated in the presence of high glucose concentrations show increased cell surface CD36 expression, as glucose enhances CD36 mRNA translational efficiency (Griffin et al. 2001). CD36 underpins insulin resistance, defective fatty acid metabolism, and hypertriglyceridemia in spontaneously hypertensive rats (Aitman et al. 1999), facilitates fatty acid uptake by cardiac and skeletal myocytes, and adipose tissues (Coburn et al. 2000), and governs triacylglycerol secretion from dietary fats (Drover et al. 2005).

CD36 also belongs to a family of scavenger receptors, instrumental in lipid flux. HDL selectively delivers cholesterol, not protein, to cells *via* the class B scavenger receptor SR-BI, a CD36 family member (Acton et al. 1996). The CD36 and lysosomal integral membrane protein II analogous-1 (CLA-1) (Calvo & Vega 1993) is homologous to SR-BI (Calvo et al. 1997) and differentially mediates LDL uptake including Ox-LDL on leukocytes, but traffics HDL in the liver (Murao et al. 1997). Targeted gene deletions of SR-A (Suzuki et al. 1997)

or CD36 scavenger receptors reduce atherosclerotic plaque size, with gene deletion of CD36 limiting plaque size more than SR-A gene deletion (Febbraio et al. 2000).

In view of the reported transcriptional regulation of CD36 by PPARγ in THP-1 monocytic cells (Tontonoz et al. 1998), it was important to assess whether LDL supplementation, particularly with ox-LDL, was capable of modulating CD36 in primary human macrophages. Cyclopentenone ligands were also used to assess the effects of PPARγ ligands on this system, and to assess whether natural arachidonates could potentially influence macrophage scavenger receptor expression.

6.1.4 Monocyte phenotypic changes relevant to vessel wall adhesion

Adhesion dictates leukocyte margination and transmigration (Springer 1994), regulating inflammatory cell recruitment to tissue injury sites (Issekutz & Issekutz 1993). Initial transient interactions underlying leukocyte capture and rolling are mediated by L-, P- and E-selectins (Puri, Finger, & Springer 1997) at respectively decreasing cell motion velocities. Integrin mediated binding underpins higher affinity binding interactions, with β_2 integrins governing leukocyte firm adhesion, and $\alpha 4$ integrins regulating both rolling and adhesion, dependent on integrin activation status. Monocyte adhesion is further influenced by extra-cellular inputs including cytokines such as MCP-1 which modulate both p150-95 and β_2 integrin expression (Jiang et al. 1992), and a critical role for β_2 integrins in monocyte transmigration has been described (Weerasinghe et al. 1998).

6.1.5 Physiologic functions of Cell Adhesion Molecules

Endothelial cells throughout the vasculature can express cell adhesion molecules (CAM). The primary focus for most inflammatory responses is in postcapillary venules, due to the high density of endothelial CAM expression, and the need to deliver effector leukocytes to sites of parenchymal injury (Butcher 1991). *In vivo* observations of leukocyte behaviour in venules have led to a model of leukocyte-endothelial cell interactions with three sequential steps for leukocyte recruitment: rolling, firm adhesion, and emigration (Springer 1990).

Intra-vascular leukocytes initially move from the central stream of flowing blood toward the vessel wall, bound by weak low-affinity adhesive interactions (Figure 6.1a). Although VLA-4, VCAM-1, MadCAM-1, and α_7 integrins have been implicated in early leukocyte rolling and tethering adhesion, the predominant interactions at this stage are between selectins and their ligands, an appearance manifest as leukocyte rolling and tethering (Lawrence & Springer 1991). Tethered leukocytes exposed to low concentrations of chemoattractants and inflammatory mediators undergo activation accompanied by L-selectin shedding. At sites of

infection or inflammatory injury, chemokines, bacterial peptides, platelet activating factor (PAF), and leukotriene B_4 act to increase integrin avidity and thus integrin-dependent leukocyte adherence (Figure 6.1b). The trans-endothelial migration of leukocytes then proceeds, with movement of adherent monocytes and neutrophils through intercellular junctions of vascular endothelial cells, a mechanism dependent upon platelet/endothelial cell adhesion molecule 1 (PECAM-1; CD31; Figure 6.1b) (Muller et al. 1993). Leukocytes continually establish new adhesive contacts at the migration front, while reducing adhesive interactions at the tail. Studies showing E- and P-selectin-dependent leukocyte rolling in mouse aorta confirm that these generic adhesion and motility mechanisms pertain to large vessel injury also, and are thus of particular relevance to atherosclerosis (Eriksson et al. 2000).

6.1.6 Leukocyte cell adhesion molecules relevant to atherosclerosis

6.1.6.1 Selectins

Selectins are a family of three differing glycoproteins. They share a common structure of an N-terminal C-type lectin domain, followed by an epidermal growth factor (EGF)-like domain, varying numbers of short consensus repeat domains, a short transmembrane portion and a cytoplasmic domain. The three selectins are categorized according to cellular distribution. L-selectin is constitutively expressed upon almost all leukocytes including T and B lymphocytes and NK cells, dependent upon activation state, and interacts with the integrin heterodimer CD11b/CD18 (Mac-1) (Simon et al. 1995). L-selectin is involved in leukocyte traffic and homing, a mechanism that involves platelet interactions (Diacovo et al. 1996). E-selectin is transcriptionally induced by NF-κB in endothelial cells under the influence of cytokine stimuli, augmenting leukocyte adhesion *via* ICAM-1 (Luscinskas et al. 1991) (see section 6.1.6.3). P-selectin is stored in resting cells in α-granules of platelets and Weibel-Palade bodies of endothelial cells and rapidly recruited within minutes to the surface of activated platelets.

Following cell activation, selectin molecules are rapidly lost from the cell surface, P- and E-selectin by internalisation and lysosomal targeting, and L-, and E-selectin by proteolytic cleavage or shedding. Circulating soluble isoforms of E-selectin and L-selectin are thus detectable in peripheral blood, soluble L-selectin acting as a further means of leukocyte recruitment (Hafezi-Moghadam et al. 2001). Soluble P-selectin results from an alternative splice lacking the transmembrane domain (Ishiwata et al. 1994), and shedding may contribute to a shorter soluble isoform of P-selectin (Berger, Hartwell, & Wagner 1998). Shed selectins may act as functional proteins, competing at their normal counter-receptors.

Selectins interact with fucosylated and sialylated carbohydrates including sialyl Lewis X, and with higher affinity mucin-like glycoprotein ligands (Kansas 1996). The major ligand for P-selectin is leukocyte P-selectin glycoprotein ligand (PSGL-1)(Larsen et al. 1989). P-selectin and PSGL-1 are a pair of extended molecules, functioning as a disulphide-bound homodimer of fucosylated and sulphated glycoproteins (Li et al. 1996). PSGL-1 is also a ligand for L-and E-selectins but with lower affinities than P-selectin (Norman et al. 2000). PSGL-1/P-selectin binding facilitates close interactions between cells, enabling other adhesion molecules like integrins to take up higher affinity interactions. P-selectin/PSGL-1 binding triggers leukocyte activation, integrin mobilization and induces inflammation and thrombosis (Evangelista et al. 1999), with antibody inhibition of PSGL-1 function reducing adhesion and recruitment on P-selectin expressing cells (Moore & Thompson 1992). Although not directly examined in the studies presented here, it is important to appreciate the role of selectins in the initiation of leukocyte adhesion, occurring prior to higher affinity interactions mediated by integrins.

6.1.6.2 Integrins

The integrin family of cell adhesion molecules encompasses heterodimeric proteins composed of non-covalently bound α and β subunits. 15 α- and 8 β-chains are currently documented. Monocyte adhesive interactions with extra-cellular matrices are mediated by integrin ligation, an event that dictates monocyte migration and extrinsic cellular signal transduction. Early investigations elucidating the nature of integrins suggested that the existence of three subfamilies within a greater family of human adhesion protein receptor heterodimers based upon the number of different beta subunits (Hynes 1987). Two key subfamilies were subsequently defined as the platelet and the endothelial cell heterodimers, which use GP IIIa, and the leukocyte heterodimers, which contain a 95,000 Da beta subunit, a protein that is distinct from, but homologous to, GP IIIa.

Conformation dictates integrin function, and is cation dependent. Conformational rearrangements of integrin extracellular domains increase ligand affinity, an effect mediated by intracellular binding of cytoskeletal talin to beta-subunit cytoplasmic tails (Tadokoro et al. 2003). Alterations in signalling competence further determine integrin function, a feature noted in Glanzmann thrombasthaenia and leukocyte adhesion deficiency-1. Despite normal levels of β-1 -2 and -3 chain surface expression, a failure of inside-out signalling in these subjects produces leukocyte dysfunction (McDowall et al. 2003). Individual integrin classes and subunits with relevance to atherosclerosis and inflammation merit closer consideration in the context of the work presented.

β1 integrins

β_1-integrins are comprised of the common β-subunit CD29, linked to a distinct very late antigen (VLA) α-subunit. The VLA family of heterodimeric cell-surface glycoproteins were originally identified on activated human T lymphocytes and named because the first such glycoproteins identified (VLA-1 and VLA-2) were expressed at a late stage of T-cell activation. Six VLA isoforms exist, each consisting of a distinct alpha chain, numbered 1 to 6. The VLA proteins belong to a superfamily of structurally and functionally related adhesion-mediating cell surface proteins that include the fibroblast fibronectin receptor (VLA5), the LFA1/MAC1 group, and the platelet glycoprotein IIb/IIIa -fibronectin receptor group. The α4β1-integrin (CD49d/CD29, VLA-4) aids leukocyte recruitment to the vessel wall, mediating the adhesion of eosinophils (Schleimer et al. 1992), lymphocytes and natural killer cells (Allavena et al. 1991), as well as monocytes, to the surface of cytokine-activated endothelial cells. Binding to relevant ligands is of importance in atherosclerosis, and direct interactions between VLA-4 and VCAM-1 have been described (Alon et al. 1995).

As with other integrin heterodimers, β1 integrin can be multi-functional. β1 integrin is involved at a structural level in multiple developmental paths. α3/β1 is required to associate with reelin in embryonic neurogenesis, (Dulabon et al. 2000), and β1 chain deletions cause fatal neuroglial malformations (Graus-Porta et al. 2001) and alter chondrocyte function obstructing normal endochondral ossification (Aszodi et al. 2003). In the immune system, β1 integrins are required for marginal B-cell localisation and compartmentalisation of peripheral lymphoid tissue (Lu & Cyster 2002).

Examples of β1 integrin function relevant to vascular biology include manganese-dependent binding of endothelial junctional adhesion molecules to lymphocyte-surface α4/β1 integrin (Cunningham et al. 2002). In the arterial media, α3β1 (a multi-functional β1 integrin) and α5β1 integrins are expressed upon VSMCs in the media of normal coronary arteries (Hillis et al. 1998). Galectin-1, a beta-galactoside-binding dimeric lectin involved in smooth muscle cell migration and proliferation, binds directly to β1 integrin in smooth muscle cells, increasing the cell surface β1 integrin copy number and inducing outside-in signalling (Moiseeva et al. 2003). Integrin β1 (ITGB1/CD29) constitutes the beta-chain of the fibronectin receptor α5β1, mediating cellular interactions with extracellular matrix. Further matrix interactions are subserved by α2β1, which binds collagen and laminin. Alterations in β1 integrin are thus critical in regulating leukocyte adhesion, but need to be investigated in relation to relevant alpha subunits, that may regulate inflammatory and atherogenic processes.

α4 integrin sub-unit

The integrin alpha chain CD49d in conjunction with CD29 forms the VLA-4 adhesion complex present on leukocytes. CD49d is involved in initial vascular wall adhesion of inflammatory leukocytes, governing transendothelial migration *via* interactions with vascular cell adhesion molecule-1 (CD106/VCAM-1), a phenomenon that is pronounced in endothelial cells that have been activated by IL-1α (Chuluyan et al. 1995). VLA-4 is a key integrin in mononuclear cell recruitment to atherosclerotic plaque in rabbit models of atherosclerosis (Kling et al. 1995). Macrophage homing to atherosclerotic plaque in murine models of atherosclerosis has been noted to be VLA-4 as well as ICAM-1 dependent (Patel et al. 1998). Intra-vital microscopic observations of leukocyte adhesion in atherosclerotic murine vessels show that α4 integrin interactions contribute more to firm adhesion than to initial tethering (Eriksson et al. 2001). Monocyte adhesion to endothelial cells that have been exposed to 12(S)-hydroxyeicosatetraenoic acid [12(S)-HETE], a product of 12-lipoxygenase metabolism of arachidonic acid metabolism, is reduced by mAb to VLA-4. The same VLA-4 dependence of monocyte-endothelial binding is also demonstrated when endothelial cells are exposed to high glucose concentrations *in vitro.* These findings suggest VLA-4 expression is important in diabetic and inflammatory vascular injury (Patricia et al. 1999), although alteration of other atherogenic stimuli including angiotensin I appears not to modulate VLA-4 expression in clinical studies (Prasad et al. 2001). Further modification of VLA-4 expression has been noted on monocytes subject to reactive oxygen species and to inflammatory mediators including arachidonic acid products, and proteases. Alteration of trafficking in this context means that areas of endothelial injury specifically recruit monocytes displaying this adhesion molecule.

α5 integrin sub-unit

The α5 integrin chain CD49e associates with β1 integrin to form VLA-5, a fibronectin receptor, expressed across leukocyte populations, including T lymphocytes (Somersalo, Carpen, & Saksela 1994) and natural killer (NK) cells (Gismondi et al. 1991). In myeloid cells, VLA-5 is involved in neutrophil adhesion, altering both Mac-1 and FcR expression following fibronectin ligation (Simms & D'Amico 1995), and assists mononuclear cell migration across fibroblast barriers, in distinction to VLA-4 assisted transendothelial migration (Shang, Lang, & Issekutz 1998). This latter phenomenon may be of relevance in the context of reparative responses to vessel wall injury in advanced stages of atherosclerosis. LDL may promote the expression of leukocyte surface VLA-5, with evidence that adsorption of LDL reduces surface monocyte VLA-5 expression in patients with occlusive arterial disease (Uno et al. 1995). Intriguingly, CD49e transcription has been

noted to be at lower levels in aortic aneurysmal tissue and occluded arterial vessels than in normal vessels in humans (Armstrong et al. 2002). Human aortic endothelial cells (HAECs) cultured with minimally-modified low-density lipoprotein differentially induces the binding of monocytes but not neutrophils, with concomitant deposition of fibronectin on the endothelial cell apical surfaces by the activation of β1 integrins associated with α5 integrins (Shih et al. 1999). The response of CD49e to ox-LDL was thus of interest in gauging potential alterations in monocyte adhesion, and would offer further insight into the relevance of β1 integrin expression profiles.

β2 integrins

The β_2-integrins consist of a common β-subunit CD18, linked to one of four α-subunits designated as CD11a, CD11b, CD11c, or CD11d, with the alpha-chain encoding genes of this integrin sub-family tightly clustered on chromosome 16 (Corbi et al. 1988). β_2-integrins are exclusively leukocytes expressed, but β_2-integrin subclass distribution varies among leukocyte subpopulations. All β_2-integrins are expressed by neutrophils, monocytes, and natural killer cells, whereas CD11a/CD18 (the lymphocyte function antigen/LFA-1) is primarily expressed on peripheral blood lymphocytes. CD11d/CD18 is moderately expressed on myelomonocytic cell lines and more strongly on tissue-compartmentalized cells such as foam cells (Van der Vieren et al. 1995). Most of CD11b/CD18 is stored in leukocyte granules (Lacal et al. 1988), where they can be rapidly (within minutes) mobilized to the cell surface after stimulation with inflammatory mediators (Freyer, Morganroth, & Todd 1989). Although CD18 was not examined in these studies, the effects of LDL and cyPGs upon relevant alpha subunits were assessed, and these subunits merit introducing individually.

αL integrin, (CD11a/LFA-1α)

Lymphocyte Functional Antigen 1 (LFA-1, CD11a/CD18) mediates leukocyte adhesion and signalling interactions, and is required for efficient T lymphocyte clonal expansion in T memory-cell driven adaptive immunity. CD11a is the unique alpha integrin subunit of LFA-1 designated αL (Sanchez-Madrid et al., 1983). Lymphocyte function-associated antigen-1 (LFA-1) shares the common β2 integrin chain CD18, with CD11b/CD18 (Mac-1) and CD11c/CD18 (p150,95). LFA-1 is expressed on lymphocytes and phagocytic cells and is involved in cytotoxic T cell adhesion to target cells. Patients with LFA-1 immunodeficiency disease (LAD) suffer recurrent life-threatening infections, with deficiency of the beta chain of all 3 molecules, LFA-1, Mac-1 (macrophage antigen-1), and p150,95, and defects in adhesion-dependent granulocyte, monocyte, and B- and T-lymphocyte functions (Marlin et al. 1986). LFA-1 is also implicated in the compartmentalisation of peripheral B cell responses, in conjunction with β1 subunits.

The relationship between alpha and beta subunits is highly important for LFA-1 function. Intracellular spatial relations between αL and β2 cytoplasmic domains are grossly altered during both intracellular activation of integrin adhesion (inside-out signalling) and extrinsic ligand binding (outside-in signalling). The dependence of bi-directional integrin signalling upon spatial interactions between cytoplasmic domains as well as extracellular conformational change, suggests cytoplasmic alpha chain levels may disproportionately dictate adhesion in the face of modest changes in cell surface LFA-1 alpha chain density (Kim, Carman, & Springer 2003).

Intravascular mononuclear cell surface CD11a levels are elevated in familial hyperlipidaemia and reduced by therapeutic lipoprotein apheresis (Rovers et al. 1998). Increases in counter-receptors for CD11a have been demonstrated in endothelial cells from atherosclerotic plaque (van der Wal et al. 1992). LFA-1 has been implicated in accelerated transplant atherosclerosis, in a manner that suggests co-dependence on ICAM-1 (Russell, Chase, & Colvin 1995). These findings all suggest that an increase in LFA-1α expression might be a response to LDL exposure in leukocytes.

αM integrin, (CD11b/Mo1)

CD11b, the αM integrin component of the β2 integrin Mac-1 (CD11b/CD18) complex, promotes the adhesion of granulocytes to each other (Ding et al. 1999) and to endothelial cell monolayers (Wetzel et al. 2004). Mac-1 contributes to firm leukocyte adhesion, (Kanse et al. 2004) and is implicated in atherosclerotic plaque progression. Recent data shows that leukocyte-platelet interactions are mediated by the inserted domain of αM, which binds platelet surface glycoprotein Ibα (Wang et al. 2005). Monocyte Mac-1 expression increases following *in vitro* exposure to ox-LDL at 100μg/ml (Terasawa et al. 2000), although this study did not utilise mAb specific to CD11b epitopes. Treatment of leukocyte donors with 600mg o.d. of alpha-tocopherol, prior to monocyte isolation and culture in ox-LDL blunted this effect, implying a critical role for LDL oxidation status in this phenotypic switch. Leukocyte phenotyping of peripheral blood samples from patients with ischaemic heart disease shows increased levels of CD11b in circulating leukocytes (Kassirer et al. 1999), although murine LDL-R-/- atherosclerotic models with further CD11b gene deletions show no obligate role for CD11b in the initiation of atherosclerosis (Kubo et al. 2000). The crucial role that CD11b plays in sequestering leukocytes to inflamed vasculature, and the specific issues raised by previous studies of CD11b expression in pro-atherogenic environments made study of this molecule an essential part of phenotyping monocyte responses to LDL.

αX integrin (CD11c/p150,95)

The alpha integrin chain CD11c (p150,95/ ITGAX), is a receptor for ICAM-1, iC3b (Myones et al. 1988) and fibrinogen. The unique CD11c alpha chain shows homology to the alpha subunits of platelet glycoprotein IIb/IIIa, the vitronectin and fibronectin receptors (Corbi et al 1987). In keeping with other integrin alpha chains that heterodimerise with β2 integrin, CD11c plays a critical role in granulocyte adhesion to fibrinogen (Loike et al. 1991) and monocyte adhesion to collagen I (Garnotel et al. 2000). Specific CD11c/CD18 binding to endothelial cell surfaces has been previously demonstrated (Stacker & Springer 1991), with implications for vascular injury. CD11c appears to be implicated in inflammatory injury during atherogenesis and plaque progression at many levels. Monocyte/macrophage production of IL-1α and IL-1β in response to LPS is dependent upon CD11c in LDL-R-/- murine models of atherosclerosis (Netea et al. 1998). CD11c positive macrophages are present in high numbers in the media of human abdominal aortic aneurysms (Koch et al. 1990), as well as being apparent in human atherosclerotic tissue (van der Wal et al. 1992). CD11c, along with CD11a, is elevated in familial hyperlipidaemia, in association with LDL-dependent upregulation of IL-1 and TNF production (Rovers et al. 1998).

One important monocyte differentiation pathway is that leading to dendritic cell morphology (Kabel et al. 1989), with the dedicated and specialised antigen presentation functions that this confers (Sallusto & Lanzavecchia 1994). Dendritic cells are highly efficient at antigen binding processing and presentation, producing immunogenic or tolerogenic responses (Finkelman et al. 1996). Dendritic cells are thus relevant to inflammatory responses in the vessel wall, especially in the context of defined auto-immune mechanisms that may involve LDL-derived epitopes. CD11c appears to be a marker of phenotypic change from monocyte to dendritic cell (Ito et al. 1999). The alteration of monocytes towards a DC phenotype has been explored in this regard, with special reference to endothelial adhesion and transmigration. DC adhesion and transmigration appear to be markedly increased after exposing ECs to hypoxia, ox-LDL or TNF-α, and by specifically inhibiting endothelial NO synthase (Weis et al. 2002). Modulation of monocyte CD11c expression by LDL or inflammatory mediators may thus offer an insight into immunoregulatory as well as adhesion and mobility changes during atherosclerosis.

β3 integrins

The β3 integrin subunit (CD61/GPIIIa) is a multi-functional adhesion molecule, and, in common with other β integrin heterodimers, recognises ligands bearing Arg-Gly-Asp-(RGD) sequences in a cation-dependent fashion (D'Souza et al. 1994). β3 integrins serve structural and developmental roles ranging from osteoclastogenesis (McHugh et al. 2000) to

placentation (Hodivala-Dilke et al. 1999) and tumour angiogenesis (Reynolds et al. 2002). β-3 integrins contribute to adhesion-dependent cell survival, with unligated β-integrin tails recruiting caspase 8 to the cell surface to act as a death receptor (Stupack et al. 2001). β-3 integrins are also central to inflammatory resolution, with the vitronectin receptor (αvβ3) enabling macrophage recognition of apoptotic neutrophils and lymphocytes (Savill et al. 1990). α2β3 integrin (glycoprotein IIb/IIIa) mediates platelet-platelet aggregation in an activation-dependent manner (Frojmovic et al. 1991). β3 integrin binds von Willebrand factor (Lefkovits, Plow, & Topol 1995), linking platelet interactions with endothelial cells (Bombeli, Schwartz, & Harlan 1998) with direct relevance to atherothrombosis and leukocytes. CD40L binds to GPIIb/IIIa in a β-3 integrin dependent manner, with binding of platelets to endothelial cells and leukocytes acting to stabilise thrombus (Andre et al. 2002).

In addition to constituting the beta-chain of the platelet glycoprotein receptor αIIβ3 and the widely expressed non-platelet integrin αvβ3, the β3 integrin subunit is important in governing cellular adhesion interactions that regulate inflammatory leukocyte responses in the vessel wall. Regulation of β3 expression is important in atherosclerotic plaque progression and is under sophisticated control mechanisms. β3-endonexin, a protein that regulates urokinase protease activator receptor (uPAR) expression through direct interaction with subunits of the NF-κB-complex also modulates β3 integrins. In contrast to uPAR or NF-κB, the expression of β3-endonexin is reduced in advanced atherosclerotic aortic tissue. β3 integrin-mediated stimulation of endothelial cells leads to enhanced NF-κB dependent secretion of monocyte chemoattractant protein-1 (MCP-1), an effect that is down-regulated by β3-endonexin (Besta et al. 2004). β3 integrins have been demonstrated in *ex vivo* samples of human atherosclerosis, with elevation of αvβ3 being particularly apparent (Hoshiga et al. 1995). Paradoxically, β3 integrins may act to limit inflammation, reducing atherosclerotic plaque: murine apo E null models of atherosclerosis show accelerated atherosclerosis in mice with gene deletion of β3 integrin (Weng et al. 2003).

Integrin ligand specificity

Integrin ligand specificity is determined by α-subunits. Two broad groups of ligands exist, the first consisting of a range of large extracellular matrix proteins including fibronectin (Sanchez-Aparicio, Dominguez-Jimenez, & Garcia-Pardo 1994), thrombospondin (Yabkowitz & Dixit 1991), vitronectin, fibrinogen, and complement component iC3b (Ramos et al. 1989). The second ligand group consists of cell surface adhesion molecules belonging to the immunoglobulin supergene family.

6.1.6.3 Immunoglobulin superfamily members

The immunoglobulin (Ig) superfamily includes a broad range of molecules with multiple Ig-like domains. Members of this family include the intercellular cell adhesion molecules-1 and -2 (ICAM-1/CD54 (Dustin et al. 1986), ICAM-2/CD102 (Staunton, Dustin, & Springer 1989)), vascular cell adhesion molecule-1 (VCAM-1/CD106) (Strauch et al. 1994), platelet-endothelial cell adhesion molecule (PECAM)-1/CD31, and the mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (Berlin et al. 1993).

The mucosal addressin MAdCAM-1 is an alternative counter-ligand to CD49d/β7 integrin and to L-selectin, expressed in the gastrointestinal tract, on high endothelial venules of Peyer's patches and embryonic lymph nodes, on small intestinal lamina propri venules and on the marginal sinus of the spleen (Streeter et al. 1988). CD31 is constitutively expressed on platelets, most leukocytes, and endothelial cells (Albelda et al. 1991).

CD106 is minimally expressed on quiescent endothelial cells, but highly upregulated by cytokine-mediated endothelial cell activation. CD106 mediates lymphocyte and monocyte adhesion in inflamed vascular beds, binding to the CD49d/CD29 heterodimer.

Endothelial CD54 is central to monocyte trafficking, binding to CD11a/CD18 and CD11b/CD18 integrins during transmigration. Basal levels of CD54 are seen on a variety of cell types, but expression is regulated on endothelial cells, (Dustin et al. 1986). CD54 expression varies between specific organs' vascular beds. Organs with relatively high constitutive expression of CD54 such as the lung exhibit smaller increments in CD54 expression after cytokine stimulation than organs with low constitutive expression such as the myocardium (Panes et al. 1995). A soluble isoform of CD54 is detectable in normal serum (Rothlein et al. 1991), with significantly elevated levels noted in disease states including viral infections (Furukawa et al. 1993) and lymphoid malignancy (Horst et al. 1991). CD11a/CD18 may also bind to CD102, a truncated form of CD54, is basally expressed on endothelial cells (de Fougerolles et al. 1991), but not upregulated on activated endothelial cells (Nortamo et al. 1991).

CD54 is involved in binding CD11a and CD11c during leukocyte margination and migration events, dictating leukocyte recruitment to inflammatory injury sites (discussed in Section 6.1.6.2). In apoE-null murine models of atherosclerosis, CD54 is highly expressed in aortic atherosclerotic lesions, in contrast to CD106 which is more weakly expressed (Nakashima et al. 1998). CD54 may be upregulated later than CD106 during atherosclerotic plaque progression, with CD106 serving early recruitment interactions to the vessel wall (Cybulsky et al. 2001). Human coronary arteries, taken from explanted hearts within 5 minutes of transplantation, show CD54 expression in focal segments of normal arterial endothelium, but

with high levels of endothelial CD54 expression apparent over atherosclerotic plaques and in macrophages, (Davies et al. 1993). *In vitro* incubation of endothelial cells with conditioned medium from mononuclear cells grown in the presence of ox-LDL enhances CD54 expression on endothelial cells, and the CD54-mediated binding of monocytoid U937 cells, suggesting a role for monocytes dictating adhesion mechanisms themselves, dependent upon specific microenvironmental influences (Frostegard et al. 1993).

6.1.7 Leukocyte migration

Leukocyte emigration through the vessel wall is determined by adhesive interactions in balance with chemoattractant stimuli that promote cell motility. Leukocytes are able to polarise (MacFarlane, Herzberg, & Nelson 1987) and move along chemoattractant gradients, in a receptor dependent manner (Nourshargh & Williams 1990). Cell motility is importantly dependent upon multiple integrin interactions including those between β2 integrin and urokinase-type plasminogen activator receptor (uPAR) (Wei et al. 1996), a system that is of specific importance to monocyte migration (Simon et al. 1996). The non-integrin receptor uPAR may bind promiscuously to multiple integrins (Simon et al. 2000c), although β2 interactions are the most comprehensively described. Loose and firm adhesion interactions with extracellular matrix components further determine the rate of progression of leukocyte motion. β1 integrin-fibronectin interactions, regulated by Rho family GTPases Rac and Cdc42, act to limit cell motility (Cox, Sastry, & Huttenlocher 2001).

6.1.7.1 Leukocyte differentiation: links to migration events

Leukocyte differentiation affects mobility and migration, due to alterations in surface adhesion molecule repertoire. Altered trafficking patterns are necessary to sub-serve particular functions including lymphocyte recirculation, antigen delivery to lymph nodes, and disposal of apoptotic and necrotic debris by macrophages. Ligation of CD31 alters leukocyte phenotype, promoting α6/β1 mediated cell migration across the vessel wall (Dangerfield et al. 2002). Defined trans-endothelial migration events direct myelomonocytic cells towards a dendritic cell phenotype (Randolph et al. 1998). Models of dendritic cell further illustrate links between motility and phenotype, with matrix metalloproteinase activity linked to Langerhans cell egress from the skin, (Ratzinger et al. 2002). The ability of monocytes to exit atherosclerotic plaque and directly re-enter the vessel lumen has been documented (Gerrity 1981b). Factors that favour atherosclerotic plaque regression including reduction of dysplidaemia promote monocyte efflux, with distinct phenotype profiles for cells that exit lesions (Llodra et al. 2004).

6.1.8 Leukocyte inflammatory function

The role of inflammatory mediators as well as lipoproteins in the initiation and progression of atherosclerotic plaque is of current interest. Oxidised LDL has been identified as a natural ligand of PPARγ (Nagy et al. 1998) contributing to plaque progression by increasing monocyte lipid uptake. Varying concentrations of LDL have been used to explore *in vitro* monocyte responses. Published data show that supplementation of medium with 50μg/ml of LDL induces a transition from a monocyte to a foam-cell phenotype (Nagy et al. 1998). The role of the nuclear receptor PPARγ in atherosclerosis has been explored with regard to foam cell phenotypic changes (Ricote et al. 1998a). The cyclopentenone-structure arachidonic acid metabolites $\Delta 12PGD_2$ and $15dPGJ_2$ have been shown to ligate PPARγ, and are produced at sites of acute inflammation. These natural PPARγ ligands reduce monocyte inflammatory responses including matrix metalloproteinase-9 production (Marx et al. 1998), relevant to extracellular matrix degradation in atherosclerosis. $15dPGJ_2$ was used to explore the effect of naturally occurring PPARγ ligands as chronic inflammatory stimuli upon monocyte/macrophages within an atherosclerotic plaque.

6.1.9 Leukocyte immunomodulatory function

6.1.9.1 Role of antigen presentation

Class II major histocompatibility complex (MHC) molecule expression on monocytes is crucial to the efficient macrophage-mediated presentation of processed antigens to T lymphocytes during adaptive cell mediated immune responses (Ziegler & Unanue 1981). The MHC coding region for the D region of human leukocyte antigen on chromosome 6 is further divided into HLA-DR, DP and DQ regions, producing the variety necessary to present multiple epitopes (Rudensky et al. 1991) to engage in specific immunological synapses (Grakoui et al. 1999). MHC Class II α and β chains are present at antigen presenting cell (APC) surfaces as heterodimers, holding peptides in facilitating peptide presentation to relevant T cell receptors (Stern et al. 1994), with the distinction from Class I MHC that antigen does not have to be endogenously processed by the APC (Morrison et al. 1986). T lymphocytes bearing a memory phenotype have been demonstrated in human atherosclerotic lesions (Hansson, Holm, & Jonasson 1989). T lymphocytes may exert regulatory as well as pro-atherogenic influences upon monocytes, with data from co-culture experiments showing that T lymphocyte derived IFN-γ reduces macrophage scavenger receptor expression, limiting lipid uptake (Geng & Hansson 1992). The possibility that chronic inflammation in atherosclerosis may be driven by an autoimmune mechanism has

been raised, with clinical data suggesting augmentation of mononuclear cell Class II expression in atherosclerotic vessels in young adults (Millonig, Malcom, & Wick 2002). Circulating autoantibodies to putative epitopes including ox-LDL-derived malondialdehyde (MDA)-lysine are found in serum, and are able to recognise material in atheromatous tissue (Salonen et al. 1992). Any alterations in Class II MHC expression in response to differentially modified LDL are thus of interest. Enhanced Class II expression might explain a process of augmented antigen presentation promoting a chronic inflammatory process in the vessel wall, due to the invocation of specific immune responses. The influence of adaptive immunity in atherosclerosis has been further explored using murine models. The initiation of atherogenesis appeared to proceed despite the absence of lymphocytes in apo E-null mice bearing a defect in recombination-activating gene 2 (RAG2), a gene that rearranges V(D)J coding regions underpinning the production of the normally varied repertoire of immunoglobulins or T cell antigen receptors. Parallel experiments showed no diminution of onset of atherosclerosis in apo E null mice with targeted gene deletions of both Class I and Class II molecules (Fyfe, Qiao, & Lusis 1994), suggesting that specific immune mechanisms may not be of importance in very early atherosclerosis.

A reduction in atherosclerotic lesional macrophage Class II expression was subsequently noted in RAG2 null animals (Daugherty et al. 1997). Class II expression is modulated during innate as well as adaptive immune responses, and is markedly enhanced by LPS stimulation (Wentworth & Ziegler 1987). Class II MHC expression is increased during monocyte activation and maturation under the influence of cytokines including GM-CSF (Hornell et al. 2003) and regulated during differentiation to specialist phenotypes including dendritic cells (Santin et al. 1999). Atherosclerotic tissue has been immunohistochemically phenotyped for Class II expression in lesions bearing high numbers of monocytes, T lymphocytes and dendritic cells (Jonasson et al. 1985), with elevated Class II molecule expression being evident in vulnerable plaques from human carotid arteries (Yilmaz et al. 2004).

Examination of monocyte Class II expression in the context of LDL and cyclopentenone exposure was thus considered an important part of monocyte phenotype analysis. Monocyte/macrophage Class II MHC expression is heterogeneous with marked variability in basal expression between individuals (Bos et al. 1990). For this reason it was essential to compare individual basal levels for each donor to calculate net expression.

6.1.9.2 Role of immune co-stimulatory molecules

Primed effector T cells are activated when complementary antigen-specific receptors and either CD4 or CD8 co-receptors bind to a relevant peptide epitope (van der Merwe & Davis

2003). However, naïve T cells require co-stimulation to initiate a proliferative response, crucial in engendering cytotoxic and memory T cell function (Chai et al. 1999). Simple ligation of the T-cell receptor and co-receptors is insufficient. To enable clonal expansion, further signals, including the ligation of CD28 and CD40, must be delivered to the surface of the recipient T lymphocyte (Somoza & Lanier 1995). The B7 molecules, structurally related homodimeric glycoproteins and members of the immunoglobulin superfamily, are present on antigen-presenting cell surfaces, with the two most extensively characterised B7 molecules being B7.1 (CD80) and B7.2 (CD86) (Bajorath, Peach, & Linsley 1994). Their natural ligand is surface-bound CD28 on T lymphocytes; contemporaneous CD28/B7 binding, along with ligation of the TCR/CD3 complex by Class II MHC in conjunction with CD4, results in T helper activation (Linsley et al. 1994).

The presence of the B7 co-stimulatory molecules has been noted in immunohistochemical analyses of murine atheroma (Lee et al. 1999) with further data suggesting that ox-LDL differentially up-regulates CD86 along with Class II MHC, in distinction from CD80 on monocytes (Fortun et al. 2001). Enhanced CD86 expression may also contribute to vascular inflammation driven by environmental factors including tobacco smoking, with evidence to suggest that nicotine augments CD86 expression in human and murine monocyte derived dendritic cells (Aicher et al. 2003). Data from murine models of atherosclerosis bearing gene deletions for LDL-R and CD80 or CD86 molecules suggests that co-stimulatory molecules are essential for early atherosclerotic progression, assisting T lymphocyte responses to HSP-60 and to ox-LDL epitopes in the first stages of plaque formation (Buono et al. 2004). Pro-atherogenic stimuli including advanced glycosylation end-products (AGE) elevated in hyperglycaemic states are associated with increased co-stimulatory molecule expression in cultured dendritic cells with concomitant stimulation of T lymphocytes, suggesting that other multiple microenvironmental stimuli may affect monocyte, or monocyte-derived cell function (Ge et al. 2005). It is likely that lymphocytes play a role in propagating atherosclerotic plaque inflammation, with lipid species acting as a potential specific epitope (Melian et al. 1999). For these reasons changes in monocyte co-stimulatory molecule expression following lipid exposure may represent an important switch in functional phenotype, enhancing chronic inflammatory responses, and merited exploration.

6.1.9.3 Monocyte receptors for immunoglobulin Fc fragments: role in inflammatory disease

Antibody mediated signalling is mediated by cell surface receptors for the Fc portion of immunoglobulin G (FcγR). The Fc ("fragment crystallisable") fragment of antibodies is the

area where complement and anti-human globulin serum (anti-IgG) bind. The Fc portion is a fundamental means of enabling the recognition and phagocytosis of microbial pathogens (Leijh et al. 1979). Monocytes express three FcγR, FcγRI, II and III, (CD64, CD32 and CD16 respectively). CD64/FcγRI exhibits myeloid lineage specific expression and acts as a high affinity receptor for IgG, reflecting monocytes' capacity for immune-complex recognition. CD64 expression is inducible by gamma-interferon, an effect mediated by a defined nucleotide sequence, the IFN-γ response region (GRR) (Pearse et al. 1993), within the gene encoding human CD64 (Pearse, Feinman, & Ravetch 1991). The removal of immune complexes, found in many inflammatory diseases, and of particular importance in type III hypersensitivity reactions (Arthus 1903) is facilitated by monocyte/macrophage Fc binding (van de Winkel et al. 1988; Van Es & Daha 1984). Recognition and ingestion of apoptotic cells during clearance and repair phases of inflammatory injury is also enhanced by Fc receptor mediated interactions with macrophages (Hogg 1987; Jungi & Hafner 1986). The binding of antibodies to leukocyte surfaces acts as a potential route for promoting auto-immune mechanisms, as macrophage recognition of Fc antibody fragments may result in localised inflammatory responses including glomerulonephritis and vasculitis in the context of inappropriate recognition of self epitopes (Matsumoto et al. 2003). Fc receptors have already been linked to C-reactive protein mediated trafficking of LDL *via* FcRγII (CD32) (Zwaka, Hombach, & Torzewski 2001). The possibility that CD64 and CD16 might be altered in monocyte exposed to LDL species, and to inflammatory mediators that may mimic the effects of modified LDL, was thus of direct interest to defining the behaviour of monocytes in pro-atherogenic environments.

CD64/FcRγI involvement in atherosclerosis

Altered lipoproteins may influence cytokine and reactive oxygen species production subsequent to CD64 ligation: hypotheses that have been postulated previously and fit with the concept of an immune-mediated pro-inflammatory stimulus for atherogenesis. Whether aggregated or altered lipoprotein might act in the same way as immune complexes is thus of particular interest. Cholesterol is necessary for normal expression of CD64 at the monocyte cell surface (Bigler et al. 1989). However immune complexes of native LDL and acetylated LDL may increase both CD64 expression and Fc receptor mediated responses in monocyte cell lines, an effect attributed to apoprotein mediated effects, as free cholesterol fails to initiate the same responses (Huang et al. 1999b).

CD16/FcRγIII involvement in atherosclerosis

The type III Fcγ receptor (CD16/FcγRIII) is the predominant FcR on cultured monocytes. CD16 exists in two isoforms encoded by separate genes, and is a glycosyl-

phosphatidylinositol (GPI) linked receptor on human neutrophil surfaces, but a type I transmembrane glycoprotein on monocytes, with less carbohydrate than its neutrophil counterpart (Edberg & Kimberly 1997). Monocyte CD16 mediates both ligand attachment and phagocytosis, with receptor expression increasing with duration of culture independent of IFN-γ stimulation (Clarkson & Ory 1988). Activation of inflammatory leukocytes rapidly mobilises CD16, with neutrophils shedding cell surface CD16 during activation, apoptosis, and chemotaxis (Tosi & Zakem 1992). Actin polymerisation enhances CD16 shedding, suggesting that the actin cytoskeleton potentially mediates the association of CD16 with integrins including leukocyte-membrane complement receptor-3 (CR3, Mac-1, CD11b/CD18) (Middelhoven et al. 1999). Soluble CD16 (sCD16) regulates monocyte inflammatory processes, binding to CR3 and inducing monocyte production of IL-6 and IL-8 (Galon et al. 1996). Elutriated monocytes stimulated by the anti-CD16 mAb 3G8 display monocyte-specific chemi-luminescent activation responses suggesting that CD16+ monocyte sub-populations mediate effector functions, including reactive oxygen species (ROS) generation, *via* CD16 ligation (Trezzini et al. 1990). Monocyte CD16 expression is altered by stimuli including LPS and IL-1 (Arend, Ammons, & Kotzin 1987), TNF-α and IL-6, IL-2 and IL-4, suggesting a role in acute inflammatory responses. Binding of LDL complexed to anti-LDL antibodies *via* CD64 and CD32 has been shown using THP monocytic cell lines and augments monocyte production of reactive oxygen species (Kiener et al. 1995). The ability of monocytes to alter CD16 expression during lipoprotein supplemented culture and maturation has not previously been explored.

6.1.10 Specific aims

The effects of *in vitro* exposure to cyclopentenones and LDL upon monocytes will be assessed.

- Alterations in monocyte ultra-structure and lipid uptake will be gauged using microscopy.
- Markers of differentiation, pattern recognition receptors, adhesion molecules and immuno-regulatory molecules will be specifically assessed.
- Differences in monocyte surface molecule expression will be examined following suspension and adhesion culture.

6.2 Results

For brevity, only statistically significant results are described in the text. Sample numbers and p values are annotated in figure legends. Full statistical analyses, and all data tables, micrographs and supplementary figures are viewable in the supplementary data CD-ROM, and in the Chapter 6 Data Appendix.

6.2.1 Blood donor characteristics

Blood was collected from donors under the auspices of local ethical approval. Donor characteristics were available for experiments to examine adherent monocyte culture and a limited number of experiments to examine responses to suspension culture. All donors were non-smokers, with no history of inflammatory or malignant disease. Further exclusion criteria were the use of anti-inflammatory agents, including inhaled corticosteroids or anti-histamines, to serve as a surrogate marker of underlying infection, inflammation or atopy. Donors who had previously been noted to be eosinophilic were excluded. Predominantly male donors were used to limit the possibility of menstruation-related platelet activation. Menstrual cycle was not ascertained during donor screening, as this was not sanctioned by the ethics committee. Due to local restrictions on use of volunteer samples serum was not permitted to be biochemically analysed in retrospect. Donor characteristics for adherent phenotyping experiments and of age, gender, and cardiovascular risk factors are presented as supplementary data in the Chapter 6 Appendix, Table 6.1.

6.2.2 Monocyte preparation and purification levels

Human peripheral blood monocytes, isolated using discontinuous Percoll gradient centrifugation, were assessed for cell purity and yield immediately after leukocyte recovery from gradients. Monocyte percentages varied between 10 and 30% of total mononuclear cells dependant on individual donors. Further specific monocyte purification was performed by a negative selection technique using immuno-magnetic bead separation, achieving purities approaching 90% (mean 89.83% +/- 0.426% s.e.m, n=60). Scatter profiles were used to determine leukocyte subpopulations by flow cytometry for the purposes of quantification of purity (Figure 6.2).

6.2.3 Ultra-structural changes in monocytes exposed to LDL

It has been shown that monocytes isolated from *ex vivo* atherosclerotic plaque demonstrate a lipid-laden appearance, with marked vacuolation representing lipid ingestion. To delineate whether differentially modified lipoproteins acted to mature monocytes in a differential

manner, micrographs were made of cyto-centrifuge preparations using monocytes matured in serum-replete media (Figure 6.3). Light microscopy suggested that ox-LDL induced increases in monocyte intracellular vacuoles (Figure 6.4), after 7 days in culture, with appearances similar to the "foam-cell" phenotype reported by other groups (Aqel et al. 1984). Vacuolation is a feature of normal macrophage maturation (Silverstein, Steinman, & Cohn 1977), and is critical to phagocytic function. Formation of phagolysosomes was apparent by transmission electron microscopy in monocytes cultured under control conditions (Figure 6.5). However, more pronounced vacuolation was visible by electron microscopy following ox-LDL exposure for 7 days (Figure 6.6).

6.2.4 Direct evidence of lipid uptake in cultured monocytes

Cultured monocytes are capable of lipid uptake *via* multiple routes, including the LDL receptor, as seen in pathological hyperlipidaemic states (Nguyen et al. 1988), as well as *via* scavenger receptors (SR) A and B (Geng, Kodama, & Hansson 1994). Total lipid uptake may be assessed visually by oil red O (ORO) labelling, non-specifically demarcating intracellular triglycerides and fatty acid chains. ORO uptake was visible in adherent monocytes cultured in both n-LDL and ox-LDL at increasing concentrations after only 24hr. Micrographs taken at low (x10) magnification indicated that ORO positive-labelling appeared similar in both n-LDL and ox-LDL supplements (Figure 6.7). Culture of adherent monocytes in control medium showed low but visible levels of ORO, which was present following prolonged monocyte culture towards a macrophage phenotype at 8 days, and confirmed by high-magnification photomicrography (Figure 6.8). Clear ORO labelling was visible in macrophages cultured contemporaneously in increasing concentrations of n-LDL at 10μg/ml, 50μg/ml, and 100μg/ml (Figure 6.9). ORO labelling of in ox-LDL supplemented macrophages at 8 days was suggestive of higher levels of ORO uptake at 10μg/ml, 50μg/ml, and 100μg/ml (Figure 6.10).

6.2.5 Evidence of specific LDL uptake by cultured adherent monocytes

Direct fluorescence labelling of LDL offers a means of monitoring the uptake of specific LDL sub-fractions, and modified LDL species (Gorog & Kakkar 1987). *In vitro* exposure of monocytes to Di-I-labelled ox-LDL resulted in intracellular fluorescence, apparent after only 24 hrs of LDL exposure, and proportional to the concentration of LDL supplementation (Figure 6.11). Nuclear counter-labelling demonstrated that these monocytes were viable, with a normal horseshoe nuclear appearance (Figure 6.12). Further exposure to Di-I labelled ox-LDL for 48hrs increased intracellular fluorescence in proportion to LDL concentration

(Figure 6.13). Monocyte culture in increasing concentrations of Di-I labelled n-LDL or ox-LDL for 8 days caused increases in fluorescence proportionate to LDL concentrations, with ox-LDL taken up more avidly than n-LDL at corresponding concentrations, as assessed by con-focal microscopy (Figures 6.14 and 6.15). Although it is possible to quantify raw fluorescence in these micrographs using post hoc software analysis, it was not possible to adjust fluorescence for cell numbers and more importantly for cytoplasmic volume per cell. For this reason, fluorescence microscopy and con-focal data are presented as qualitative representations of intra-cytoplasmic LDL uptake.

6.2.6 Alteration of monocyte flow cytometric properties

Flow cytometric properties of monocytes in suspension did not appear to differ when comparing cells cultured in control media to those cultured in the presence of n-LDL. However, alterations in side and forward scatter appeared within 24hrs of suspension culture in the presence of ox-LDL at high concentrations (Figure 6.16). The differential effects of ox-LDL did not appear to be present following prolonged culture (Figure 6.17). Prolonged suspension culture with the cyPG $15dPGJ_2$ did not appear to alter scatter profiles (data not shown). For acquisition of data for adherent cultures altered scatter properties necessitated a switch to logarithmic scale (Figure 6.18). Gating for monocytes was undertaken in this manner, with further gating using the FL-4 channel for ToPro3 fluorescence to exclude dead cells (Figure 6.19). The fluorescence for the gated population was represented as a histogram with median fluorescence being used for quantification. Alteration of monocyte auto-fluorescence was noted following exposure to ox-LDL in suspension culture for prolonged periods, with evidence that basal fluorescence of unlabelled cells rose differentially, but varied between donors (Figure 6.20). This necessitated the subtraction of basal fluorescence signals during of flow cytometric quantification analysis.

6.2.7 Molecular surface phenotyping of monocytes: pattern recognition receptors

6.2.7.1 Monocyte CD14 expression following LDL and cyclopentenone exposure

For these experiments, serum-replete monocytes were cultured in suspension with native or oxidised LDL, or with cyclopentenone prostaglandins across a 3-log concentration range. Monocyte surface CD14 expression appeared unchanged in response to supplementation with both n-LDL and ox-LDL at increasing concentrations during suspension culture at 24hrs, 72hrs and 5 days (Figures 6.21-6.23). CD14 expression remained unchanged during

suspension culture with at 24hrs, 72hrs and 5 days in medium supplemented with $15dPGJ_2$ (Figure 6.24). No alteration of monocyte CD14 was noted during adherent culture in the presence of 50μg/ml of n-LDL and ox-LDL at 48hrs (Figure 6.25). However, at 8 days 50μg/ml n-LDL significantly reduced CD14 expression (n=8, p=0.0303, net median fluorescence, n=8, p=0.4727, net fluorescence relative to control). Ox-LDL at the same concentration had no effect upon CD14 expression (Figure 6.26).

For CD14 data tables, see supplementary data (Chapter 6 Appendix, Tables 6.2-6.9).

6.2.7.2 Monocyte CD36 expression following LDL and cyclopentenone exposure

Monocyte CD36 was not significantly altered by culture in LDL supplements, during suspension culture at 24hrs, 72hrs or at 5 days, (Figures 6.27 to 6.29). Furthermore, no significant alteration of CD36 expression was noted during suspension culture with increasing concentrations of cyclopentenone $15dPGJ_2$ after 24hrs, 72hrs or 5days (Figure 6.30), although the later time points suggested lower levels of expression. This could not be explained by cell death, in view of live-gating to exclude ToPro3 positive cells.

Molecular phenotyping of CD36 in adherent monocytes culture with 50μg/ml of n-LDL and ox-LDL showed no significant alterations at 48hrs or 8 days (Figures 6.31 and 6.32), again suggesting stable monocyte CD36 expression, despite LDL supplementation. A non-significant trend towards decreased CD36 expression in adherent macrophage cultures was noted after 8 days of ox-LDL exposure.

For CD36 data tables, see supplementary data (Chapter 6 Appendix, Tables 6.10-6.17).

6.2.8 Molecular phenotyping of monocytes: adhesion molecules

6.2.8.1 Monocyte surface CD29 (β1 integrin) expression is unaltered by lipid exposure

Flow cytometric analysis showed stable monocyte β1 integrin expression following exposure to increasing concentrations of n-LDL and ox-LDL at 24hrs, 72hrs and 5 days in suspension culture. 24hrs of culture with $15dPGJ_2$ caused no alteration in CD29 expression. Prolonged culture with higher concentrations of $15dPGJ_2$ showed a linear trend to decreased total CD29 expression at 72 hrs, (one-way ANOVA, n=3 p=0.0099), and a significant down-regulation of CD29 relative to control at 72hrs (one-way ANOVA, n=3, p=0.0259) but not at 5 days.

Limited samples were available for assessment of monocyte phenotype in response to LDL supplements at 48hrs in adherent culture, but representative flow cytometry data suggested that β1 integrin expression was not markedly altered, with no further change seen on

representative plots after 8 days of adherent culture. However, the latter are qualitative data given the insufficient sample numbers that preclude formal statistical analysis. CD29 data plots histograms and tables are presented as supplementary data, (Chapter 6 Appendix, Figures A6.1-A6.6, Tables 6.18-6.24).

6.2.8.2 Adherent monocyte CD49d expression is reduced by ox-LDL

Adherent culture of monocytes on tissue culture plastic in serum-replete conditions with LDL supplementation did not statistically alter net median fluorescence. However, ox-LDL appeared to produce a statistically significant decrement in CD49d expression relative to control at 48hrs (Wilcoxon signed rank test, n=7 p=0.0391, see Figure 6.33), although this was not obvious from histogram overlays. One-way ANOVA did not show changes of total net median fluorescence following LDL treatments (n=7 p=0.0515), but 50μg/ml ox-LDL appeared to reduce net median CD49d fluorescence relative to control at 8 days (Wilcoxon signed rank test, p=0.0156, n=7, Figure 6.34). CD49d expression was not assessed on suspension culture monocytes. The histograms suggest that this alteration in fluorescence is small in absolute terms. Overall, when raw fluorescence data were viewed, CD49d signals were at the lower range of the fluorescence scale, and changes were not gross despite statistical analysis suggesting significance. For full data analysis, see supplementary data, Chapter 6 Appendix, Tables 6.25 and 6.26.

6.2.8.3 Monocyte surface CD49e expression following lipoprotein exposure

Adherent but not suspension cultured monocytes were available for CD49e expression quantification, but no statistically significant changes were noted following 48hrs or 8 days in culture supplemented by 50μg/ml of n-LDL or ox-LDL. Responses to cyclopentenones were not measured for CD49d or CD49e. (See supplementary data, Chapter 6 appendix Figure A6.7 and A6.8, and Tables 6.27 and 6.28).

6.2.8.4 Monocyte surface CD11a expression following LDL and cyclopentenone exposure

Investigation of CD11a expression was initially explored in the context of transcriptional responses to LDL in Chapter 5. The discrepancies between transcriptional changes and alterations at a protein level were discussed in Chapter 5, section 5.4.4.

Monocyte surface CD11a expression was unchanged by LDL supplementation in suspension culture at 24hrs, 72hrs and 5 days. No statistically significant alteration of CD11a expression was noted at the same time points following exposure of suspension cultured monocytes to increasing concentrations of the cyclopentenone $15dPGJ_2$. The issue of differential adhesion-

dependent LFA-1 expression was addressed by assessing mature monocyte/macrophage CD11a surface expression after culture on tissue culture plastic, followed by detachment. Although a statistically significant reduction in monocyte CD11a surface expression relative to control was noted at 48hrs, histograms showed little reduction in actual fluorescence. No changes were noted at 8 days in adherent culture. For CD11a data plots, histograms and data tables, see supplementary data, Chapter 6 Appendix, Figures A6.9-A6.14, and Tables 6.29-6.35.

6.2.8.5 Monocyte surface CD11b expression following LDL exposure

CD11b expression on monocytes in suspension culture was assessed at 24hrs only, with no significant changes noted (Figure 6.35). Early assessment of surface CD11b expression on adherent monocytes after 48hrs revealed a non-significant enhancement of expression in monocytes exposed to n-LDL at 50μg/ml, but no significant changes in monocytes exposed to ox-LDL at 50μg/ml (Figure 6.36). However, 8 days of exposure of adherent monocytes to ox-LDL at 50μg/ml significantly reduced net median CD11b monocyte surface fluorescence ($n=9$, $p=0.0029$) and net median CD11b fluorescence relative to control ($n=9$, $p=0.002$), an effect not reproduced by n-LDL, (Figure 6.37). Ox-LDL also produced a significant decrement in CD11b compared to n-LDL ($n=9$, $p=0.0003$). A divergent change was apparent, with certain donors showing marked reduction in CD11b expression. Overall, this may suggest differential control of monocyte CD11b expression dependent upon maturation and adhesion status. For full data analysis, see supplementary Tables 6.37 and 6.39.

6.2.8.6 Monocyte surface CD11c expression following LDL and cyclopentenone exposure

Suspension culture showed no statistically significant changes in monocyte surface expression of CD11c in response to LDL supplementation at increasing concentrations at 24hrs (Figure 6.38). A significant reduction in total net median CD11c appeared to be induced by ox-LDL at 100μg/ml following 72hrs of LDL-supplemented suspension culture, but this was not apparent when relative fluorescence was compared to control (Figure 6.39). No alterations in CD11c were seen at 5 days in LDL-supplemented suspension culture (Figure 6.40). Furthermore, no alteration in CD11c expression was noted in response to cyclopentenone $15dPGJ_2$ exposure at the same time points (Figure 6.41). Adherent culture of monocytes in n-LDL or ox-LDL at 50μg/ml showed no statistically significant alterations in CD11c expression at 48hrs (Figure 6.42), but a marked and statistically significant reduction in ox-LDL in comparison to control treatments and n-LDL was noted at 8 days ($n=9$,

p=0.002 and p=0.0024 respectively, see Figure 6.43). This suggests that a further alpha integrin subunit alters expression in response to LDL exposure, dependent upon maturation and adhesion status. (For full data analysis, see supplementary data, Tables 6.40-6.47).

6.2.8.7 Monocyte CD61 (β3 integrin) expression in response to LDL

Monocytes surface β3 integrin expression was assayed by indirect immunofluorescence using anti-CD61 mAb. 50μg/ml n-LDL, but not ox-LDL, significantly reduced CD61 expression relative to control at 48hrs (Wilcoxon signed rank test, p=0.0391, n=7) but not at 8 days. However, the actual reduction in fluorescence was small. CD61 expression relative to control appeared significantly reduced by 50μg/ml ox-LDL but not by n-LDL at 8 days (Wilcoxon signed rank test, n=7, p=0.0078). Absolute changes of fluorescence were low, with signals being at the lower end of the cytometer range, making interpretation of these data difficult. Suspension culture phenotyping was not performed using this mAb. CD61 data plots, histograms and data tables, are presented as supplementary data, Chapter 6 Appendix, Figures A6.15 and A6.16, and Tables 6.48-6.49.

6.2.8.8 Monocyte CD54 (ICAM-1) expression in response to LDL

Monocyte CD54 was assessed in suspension culture at 24hrs in 50μg/ml of n-LDL or ox-LDL supplemented media, and a statistically significant increase in expression noted in response to ox-LDL, relative to both control and to n-LDL (n=6, p=0.0156, and p=0.0022 respectively, see Figure 6.44). Insufficient samples were available for statistical analysis at later time points in suspension culture, but representative flow cytometric histograms suggest that CD54 expression may be unaltered relative to control once suspension culture monocytes have matured at 5 days (Figure 6.45).

Assessment of adherent monocytes in n-LDL or ox-LDL at 50μg/ml showed no alteration in CD54 immunofluorescence at 48hrs (Figure 6.46). However, protracted adherent culture for 8 days in 50μg/ml of ox-LDL significantly reduced both net median CD54 expression and net CD54 expression relative to control (n=7, p=0.0272 and p=0.0379 respectively, see Figure 6.47). These data suggest that differential expression of this immunoglobulin superfamily member following LDL exposure is dependent upon adhesion status. (For full data analyses, see supplementary data, Chapter 6 Appendix, Tables 6.50 to 6.52)

6.2.9 Molecular phenotyping of monocytes: immunoregulatory molecules

6.2.9.1 Monocyte CD64 (FcγRI) expression following LDL and cyclopentenone exposure

Although assessment of suspension culture monocytes at 24hrs suggested a significant reduction in CD64 in Ox-LDL treated monocytes compared to n-LDL treated cells at 100μg/ml (n=4 to 9, p=0.0117), this was not corroborated when comparing net changes in fluorescence relative to control at 24hrs. Exposure of suspension-cultured monocytes to n-LDL or ox-LDL caused no statistically significant changes in CD64 expression at 72hrs, or 5 days. Stable CD64 expression was seen following exposure of suspension cultured monocytes to increasing concentrations of $15dPGJ_2$, although a trend towards reduced expression was noted with 10μM $15dPGJ_2$ after 5 days culture. However, the data are non-significant, suggesting that PPARγ is uninvolved in this phenotypic change. Monocytes in adherent culture exposed to 50μg/ml of n-LDL or ox-LDL showed no alteration of CD64 expression after 48hrs or 8 days. These data suggest that CD64 expression is unaltered by ox-LDL exposure.

For CD64 data plots, histograms and data tables, see supplementary data, Chapter 6 Appendix, Figures A6.17-A6.22, and Tables 6.53 to 6.60).

6.2.9.2 Monocyte CD16 (FcγRIII) expression following LDL exposure

Phenotypic assessment of CD16 expression was performed on cells in adherent culture alone. Exposure of adherent monocytes to 50μg/ml n-LDL or ox-LDL caused no statistically significant changes in surface CD16 expression at 48hrs or 8 days. The stability of CD16 expression suggests that LDL immune complex binding may not be greatly enhanced in mature lipid laden macrophages, a hypothesis that would need formal testing using aggregated LDL and anti-LDL monoclonal antibodies. For CD64 data plots, histograms and data tables, see supplementary data, Chapter 6 Appendix, Figures A6.23 and A6.24, Tables 6.61 and 6.62.

6.2.9.3 Monocyte Class II MHC expression following LDL and cyclopentenone exposure

Monocyte Class II expression was not significantly altered by culture in LDL supplements, during suspension culture at 24hrs, 72hrs, or 5 days. No significant alteration of Class II expression was noted during suspension culture with increasing concentrations of cyclopentenone $15dPGJ_2$ for up to 5 days. Adherent monocytes cultured with 50μg/ml of n-

LDL showed no significant alterations of Class II expression at 48hrs or 8 days. Ox-LDL did not alter net median Class II fluorescence but appeared to reduce Class II expression relative to control after 48hrs of adherent culture (Wilcoxon signed rank test, n=7, p=0.0156). This was not seen following 8 days of culture. Absolute fluorescence did not appear grossly reduced on histograms, although elevated isotype control fluorescence was seen as a function of ox-LDL auto-fluorescence (see Figure 6.20). (For Class II MHC data plots, histograms and data tables, see supplementary data, Chapter 6 Appendix, Figures A6.25-A6.30, Tables 6.63- 6.70.

6.2.9.4 Monocyte CD86 (B7.2) responses to LDL supplements

Initial lipid exposure caused no statistically significant change of monocyte CD86 expression in either n-LDL or ox-LDL treated monocytes at 24hrs: although the Kruskal-Wallis ANOVA test suggested a significant variation in CD86 expression (n=4 to 12, p=0.0201), Dunn's multiple comparison test revealed no significant difference in expression from control. Furthermore, no changes were noted relative to control monocyte CD86 expression (n=4-12, p=0.1708), suggesting no real variation in signal. No changes were noted with LDL exposure at 72hrs, or 5 days in suspension culture. Further phenotyping of monocytes in adherent culture showed no significant alterations in CD86 expression following exposure to n-LDL or ox-LDL at 50μg/ml for 48hrs or 8 days.

For CD86 data plots, histograms and data tables, see supplementary data, Chapter 6 Appendix, Figures A6.31-A6.35, Tables 6.71- 6.75.

6.3 Summary: monocyte phenotype changes

LDL alters monocyte surface molecular phenotype in a manner that is dependent upon both LDL oxidation status, and also upon monocyte adhesion status.

Monocyte CD14 expression was maintained during exposure to n-LDL, ox-LDL and cyclopentenones in suspension culture, but appeared reduced following prolonged exposure to n-LDL in adherent culture.

Monocyte scavenger receptor BI expression as assessed by CD36 phenotyping was statistically unchanged by culture with LDL and cyclopentenone supplements.

Ox-LDL supplementation significantly reduced levels of monocyte surface CD11b, CD11c and ICAM-1 during prolonged adherent but not suspension culture. Statistical analyses suggested that CD49d expression was reduced following prolonged adherent culture with ox-LDL. Changes in CD11a and β3 expression were noted on statistical analysis, but did not appear marked when assessing fluorescence histograms.

No significant alteration of FcγRI or FcγRIII receptor expression was noted.

A transient reduction in Class II MHC molecule expression was noted during early adherent culture, but this was not reflected by histogram appearances. B7.2 co-stimulatory molecule appeared stably expressed during culture with LDL and cyclopentenones.

Exposure to pro-atherogenic stimuli thus promotes a monocyte phenotype that potentially confers altered adhesion properties. Atherosclerosis plaque progression may be affected by this, due to limited monocyte mobility within the vessel wall. Detailed discussion of the biological implications of these findings is entered into in section 6.5.

6.4 Technical and experimental issues and study limitations

6.4.1 Use of *in vitro* models in atherosclerosis research

The ability of individual leukocyte populations to differentially traffic and vary in direct response to defined stimuli governs the plasticity of the inflammatory responses, tailoring such cellular reactions to benefit the host. Inappropriate invocation of cell surface changes may cause disruption of such cell movements, and cause the unwanted retention or conversely egress of cells from an inflammatory site.

Monocyte culture was undertaken in two different manners: suspension cell culture in Teflon containers, and adherent cell culture on tissue culture plastic. Both methods utilised highly purified cells, isolated either by immunomagnetic separation, or by attachment to tissue culture plastic and washing to remove contaminant lymphocytes and granulocytes. Clearly no *in vitro* system will represent a parallel to *in vivo* physiological conditions, but defined observations of cell behaviour may be made in this way.

Alternative animal models are widely used in atherosclerosis research. Induction of marked hyperlipidaemia has been possible with targeted gene disruption of lipid trafficking molecules. LDL-R null animals have high circulating levels of LDL, but use of the Apo E-/- murine model currently predominates, with the morphology of the atherosclerotic plaque thus produced being widespread through the vascular tree and bearing similar cellular composition to human atheroma (Nakashima et al. 1994). However, murine atheroma shows distinct differences from human atheroma (Meir & Leitersdorf 2004). Murine gene deletion models show rapid time courses of plaque progression in comparison to humans (Hockings et al. 2002), but with a predominance of plaque only in larger arterial vessels. Indeed, even very early studies demonstrated differences in plaque distribution from human disease (Wissler & Vesselinovitch 1968). Murine genetic strain differences dictate differential leukocyte responses contributing to atherosclerotic plaque formation, with C3H backgrounds

conferring protection, and C57/BL6 backgrounds promoting susceptibility to atheroma formation (Liao et al. 1994).

By contrast, the use of human *ex vivo* samples for immediate phenotyping presents problems of tissue sampling, adequate sample yield and in the case of monocytes the limited ability to examine end-differentiated cells.

6.4.2 Normal distribution of cell molecules across populations

The variability seen during molecular phenotyping is not surprising, given the donor heterogeneity that will be present in any study of human primary leukocytes, perhaps reflecting differential maturation status in circulating cells (Dransfield et al. 1988). Studies examining surface molecule expression on granulocytes have highlighted phenotypic variation in association with age, gender pregnancy stress and race (Elghetany & Lacombe 2004). Given this inherent variation of surface molecule expression in circulating primary leukocytes, the use of such cells in any phenotyping assay might be questioned. However, the advantages of primary human cells outweigh such issues, because of the limitations of using cell lines or animal models. Certainly in other models of inflammation, including airways (Galli 1997) and renal injury, murine models do not offer a full reflection of human disease processes. Although many homologous genes governing leukocyte function are present in the murine genome, fundamental differences in inflammatory responses, such as the variability in IL-8 receptor homologues in mice (Bozic et al. 1994), mean that the use of animal models must always be carefully considered. The use of selected donors for the data presented is thus an attempt to ensure a relatively homogeneous cohort to limit confounding variables that might alter surface molecule expression. Characteristics were only available for a limited number of donors, but are reflective of all donors used in these studies. Non-smokers with no recent history of inter-current infection or inflammatory symptoms were targeted, and the lack of prescribed medication was taken as a surrogate marker of good health. This donor profile is in keeping with other published work in the field of innate immunity and inflammation research.

6.4.3 Cell culture: suspension vs. adherent culture

Assessment of suspension cells offers insight into non-adherent monocyte behaviour, modelling leukocyte behaviour prior to vascular wall tethering events. The use of prolonged periods in suspension culture may be criticised. It is debatable whether uncommitted monocytes circulate for more than 72 hours *in vivo*. Data from murine models shows that circulating monocyte have a half-life of 17.4hrs (Van Furth, Diesselhoff-den Dulk, & Mattie 1973), with a half-life of three times this value in humans (van Furth 1989). This calls into

question the relevance of *in vitro* assays that examine monocyte behaviour beyond this time point. Suspension cultured cell numbers fall due to constitutive apoptosis, autophagy (Klionsky & Emr 2000), and presumed phagocytosis of effete cells, producing a potentially biased phenotype. Use of Teflon culture introduces the possibility of anoikis, with cell death due to a lack of adhesion-mediated leukocyte activation (Frisch & Francis 1994). High monocyte purity greatly reduced populations of lymphocytes and granulocytes that would undergo early apoptosis, suggesting that the monocyte phenotypic data presented here is realistic. Despite this, no clear pattern of phenotypic change appeared in the cells sampled, when data were subject to statistical analyses.

Adherent monocyte responses partially govern the progressive changes in monocyte surface molecule during the transition from intra-luminal to resident inflammatory cells. Suspension culture fails to mimic these inputs directly. To explore the specific effects of adhesion upon monocytes exposed to LDL, further phenotypic analysis was performed upon monocytes adherent to tissue culture plastic. Although this is by definition an artificial environment, it avoids the limitations inherent in suspension culture. Nevertheless, harvesting of monocytes from tissue culture plastic introduces further experimental issues. The use of trypsin to detach cells was avoided to minimise disruption of molecules that might be enzymatically cleaved from monocyte surfaces. Cells were detached in EDTA on ice followed by agitation of tissue culture flasks. Viability of cells labelled for immunophenotyping was maintained by the addition of autologous plasma to detachment buffers, and demonstrated utilising membrane permeability dyes and Annexin V to assess phosphatidylserine exposure during flow cytometry. Despite the effects these methods might have upon integrin conformation it appeared not to alter cell survival, or mAb binding of monocyte surface integrins. Also, in contrast to mechanical means of cell harvesting including detaching cells with a rubber spatula, EDTA mediated detachment minimised cell death but at the expense of lower cell yields.

It must be noted that "two-dimensional" tissue culture in only one horizontal plane fails to reproduce the three-dimensional structure that governs monocyte differentiation *in vivo* (Hakkert et al. 1990). Integrin mediated adhesion stimuli alter cytoskeletal filament tension, changing cell functions (Maniotis, Chen, & Ingber 1997). Gene transcription is critically altered by distinct adhesion steps in 3-D models, with differential effects introduced by culture on individual matrix components in gel form (Li et al. 1987). Further exploration of LDL induced phenotypic changes would thus necessitate the use of matrix components in simple three-dimensional structures such as collagen gels.

6.4.3.1 Cell culture: use of serum-replete media

Cell surface changes are an early manifestation of physiological responses to external stimuli in circulating and marginating leukocytes. In the absence of serum monocytes may survive *in vitro* when cultured adherent to plastic but die rapidly in suspension culture, following a pattern of cell survival dictated partially by integrin ligation (Frisch & Ruoslahti 1997). Furthermore, serum-replete culture facilitates the maturation of monocytes in a concentration dependent manner, permitting the *in vitro* expression of CD16 and CD51 (the alpha chain of the vitronectin receptor), in distinction from serum-free matured monocytes, which demonstrate limited expression of markers such as CD14 (Andreesen et al. 1990).

Cells cultured in a serum-replete system, although not entirely representative of the intra vascular milieu, do serve as a model for the observation of such events *in vitro*. The presence of serum allows for cellular survival, with specific factors including CSF-1 (Becker, Warren, & Haskill 1987) and upregulation of heat shock proteins (Lang et al. 2000) preventing monocyte apoptosis at critical times during monocyte differentiation. This offers the opportunity to study early cell surface responses by naive undifferentiated monocytes in order to discern the characteristic changes that dictate transition into a foam cell. The fact that normal serum contains lipoproteins at varying concentrations has not been discounted. However, autologous serum was used at 10% by volume, and clear differences were noted following modified LDL exposure relative to control. It is thus reasonable to view these studies as representing an augmented effect of modified LDL upon serum-replete monocytes. Alternative strategies would have entailed the use of heat-inactivated delipidated human serum. This is costly to produce in large quantities, and was not available for these studies.

6.4.4 Microscopy: problems assessing monocyte lipid uptake

Cultured monocytes were unequivocally demonstrated to take up intra-cellular lipid, as demonstrated by both ORO labelling and fluorescent labelling using 3,3'-dioctadecylindocarbocyanin-iodide (Di-I) LDL species. Although studies have previously shown that monocyte adoption of a lipid-laden phenotype *in vitro* requires protracted periods of culture of up to 14 days (Lesnik et al. 1992), it is clear from the data presented in this chapter that lipid uptake is initiated much earlier. However, quantification of such lipid uptake introduces further technical challenges. Micrographic analysis proved too inconsistent to be of value. Attempts were made to assess hue, saturation and intensity for each cell using both commercial OpenLab software and ImageJ open-source software (Abramoff, Magelhaes, & Ram 2004). Correlation of LDL labelling with each cell was not

possible, as cell numbers were underestimated. Indirect assays including the use of radio labelled LDL species have been previously used to assess leukocyte lipid uptake (Jones, Reagan, & Willingham 2000). Replicating this method would have necessitated further sourcing of different LDL samples, introducing potential inconsistencies with the original data produced. Also this method would have correlated radioisotope activity to cell protein content, but would not specifically address the issue of lipid uptake per cell. Thus alterations in cell size, changing protein concentration in lysed samples, would only confound assessment of cell numbers per sample.

Other options for quantifying lipid uptake are the use of ORO labelling, followed by spectrophotometric analysis for colour content, producing a surrogate marker of lipid uptake (Wilsie et al. 2005). Such analysis would not produce a specific measure of LDL uptake, neither would it accurately define lipid uptake per cell, offering only an average reading based on the assumption that cell numbers were constant in each treatment.

The data presented regarding monocyte for lipid uptake are thus qualitative, but demonstrate a number of factors. Firstly, all monocytes cultured in serum-replete conditions were exposed to low-levels of lipids in multiple forms, including triglycerides and cholesterol esters, a factor made apparent by ORO labelling seen in control cells. Secondly, LDL uptake increases with duration of culture, and maturation of monocytes, an observation that is reflected by increased ORO labelling indicating total lipid uptake and the increased specific LDL uptake observed during prolonged culture with Di-I LDL. Thirdly, the type of LDL supplementation dictates the extent of total lipid uptake, with enhanced ORO labelling seen in cells treated with ox-LDL in contrast to controls and n-LDL treated cells.

6.4.5 Quantification of indirect immuno-fluorescence

Quantification of indirect immuno-fluorescence requires careful consideration. The use of controls in particular is a potential source of error. Isotype matched non-specific monoclonal antibodies are often used to distinguish background antibody labelling, an issue relevant to work with myeloid cells bearing surface Fc receptors at a biologically important molecular copy number. However, the use of isotype controls as a definitive mode of quantification raises further issues (Keeney et al. 1998). The saturation of individual isotype controls needs to be assessed in relation to individual antibodies. Protein concentrations from manufacturers may vary from batch to batch, necessitating the titration of isotype non-specific binding on each occasion. Isotype controls from each experiment would thus have to be titrated to the same fluorescence level as previous batches. In the case of isotype controls, this single variable is supposedly the specificity of the isotype for the epitope. Each monoclonal needs

to be matched to an isotype control at the same protein concentration, to ensure a rational baseline non-specific binding. In experiments with multiple samples being analysed for the expression of multiple molecules with multiple antibodies, this may necessitate the use of considerable numbers of isotype control dilutions. Issues regarding variability of the fluorochrome to protein ratio are also problematic when using isotype controls as a quantification tool, although this is clearly more relevant to pre-conjugated antibodies. The work presented in this chapter utilised un-conjugated monoclonal antibody labelling, detected with saturating concentrations of anti-mouse immunoglobulin $F(ab)_2$ fragments conjugated to FITC. Variable primary antibody:fluorochrome ratios were thus avoided. However, batch variability of secondary antibody remains a theoretical source of inconsistent labelling between experiments. Any isotype control will by definition represent a different peptide sequence to the test antibody, and the variation in amino acid sequence raises the theoretical possibility of selective and differential binding to Fc receptors. Isotype labelling is important to indicate whether an issue with background non-specific antibody labelling exists or not (O'Gorman & Thomas 1999). In the absence of an alternative control, it has been used in the experimental data presented. However, examination of elevated signals from most of the experimental samples analysed suggests that different isotype binding characteristics were not consistently seen when IgG_1 and IgG_{2a} binding were compared, and then further compared to unlabelled cells. A more robust approach might be the use of a cell population bearing all but the molecule of interest, the "fluorescence minus one" method (Roederer 2001), of use in multi-fluorochrome analyses where controls display all but the molecules under examination on the cell surface. Quantification still remains unresolved even with this technique, and this might best be dealt with by utilising control fluorescent microspheres with defined fluorescence profiles, although these quantification controls must be carefully prepared (Gratama et al. 1998). Microspheres could be used to calibrate individual cell populations, enabling quantification data to be compared across multiple experiments.

Isotype control labelling draws attention to overtly high levels of non-specific mAb binding, a phenomenon that is useful when examining myeloid cells with high levels of FcR, especially in murine systems. Should non-specific binding then appear unacceptably high experiments may be redesigned, altering blocking steps used before mAb labelling, or subsituting different mAb altogether. However, the use of isotype controls as absolute quantification tools must be regarded as limited in any large scale flow cytometric assessment that involves multiple samples over a protracted period of time.

6.4.5.1 Relative fluorescence assessment

In view of the discrepancies in baseline fluorescence signals between different cell preparations, it was necessary to devise a practical solution to address variable controls. Whilst a variation in control fluorescence may not appear to be numerically important, the alterations of high signals on a log scale are critical. Thus removal of control values is necessary for precise quantification. The adopted method of removing isotype control signals was used for each sample. The control treatment signal was then further subtracted to give a net change in fluorescence. Analogous "delta-delta" methods are used in gene array assessments of variable background control signals, and have been recommended for flow cytometric quantification in the absence of calibrated signals (Mario Roederer, pers. comm. 2005). The definitive use of fluorescent beads would avoid the need for such a strategy (e.g. Rainbow beads, Spherotec Inc., IL, USA). Utilising calibrated beads for each assay would establish a relative fluorescence control level, enabling calibration of each cell for each individual donor. Relative fluorescence could then be calculated in the same manner, with the advantage of gaining an absolute control. Calculation of absolute numbers of molecules per cell becomes possible with this technique, enabling true quantification of cell surface copy number. It is unusual in experimental immunology to routinely use calibration beads for such purposes, although their use in clinical phenotyping is important, especially for monitoring progress of CD4 cell counts in acquired immune deficiency and the responses to chemotherapy in myeloid neoplasia (Gratama et al. 1998). In the absence of such calibration beads the data presented still represents relative variation in molecular expression, but a move to absolute quantification offers an additional level of strength to such experiments, and would be used in future work.

6.4.5.2 Auto-fluorescence

Monocytes exposed to LDL in both suspension and adherent culture demonstrate auto-fluorescence in a manner that appears to increase according to the type of LDL species used to supplement culture media, and the duration of culture. Parallels may be drawn here with other macrophage flow cytometric analyses, including the phenotypic evaluation of alveolar macrophages (Lehnert et al. 1986). LDL mediated alteration of auto-fluorescence in leukocytes has been previously noted (Schmitz et al. 1993), and relates to macrophage ingestion of ox-LDL aggregates bearing conformational changes caused by creation of Schiff-base structures (Maeba, Shimasaki, & Ueta 1994). Quantification of relative fluorescence changes in cell culture systems with high-levels of auto-fluorescence is difficult when comparing changes with similar cell treatments that do not show the same alteration in

intrinsic fluorescence. LDL uptake is also problematic in that auto-fluorescence may be diminished paradoxically, thus producing apparent decrements in cell fluorescence for all markers in specific cell culture conditions.

6.4.5.3 Statistical analyses of *in vitro* phenotyping data

Appropriate statistical tests have been carefully chosen to interrogate the flow cytometric data produced in this chapter, after discussions with an epidemiologist and a statistician. Although normality tests may be utilised to explore the distribution of data sets, sample groups with numbers less than 12 cannot be deemed large enough to reliably use such tests (Dallal & Wilkinson 1986). For this reason, non-parametric assays have been used throughout. This may have reduced the likelihood of significant difference being highlighted during analysis.

Changes in fluorescence were assessed using non-parametric analogues of ANOVA tests for data sets with more than two groups. Data pairing was checked prior to calculations using Spearman coefficient calculations. For data sets that showed poor data pairing, or where p values were not calculable with paired data, the Kruskal-Wallis test was utilised. The Friedman test was utilised for data sets showing effective pairing. Dunn's multiple comparisons were performed on all data where significant differences were demonstrable and individual p values for comparisons are shown in data tables. Comparisons of net fluorescence with a theoretical value of zero were not performed as this would have necessitated multiple t testing, with the chance of introducing a type II error.

Two-tailed t tests were used for assessment of data sets with two groups. Variation of these groups from a theoretical value of zero was necessary when assessing fluorescence relative to control and was performed using Wilcoxon signed-rank testing. Where data matching was ineffective, the Mann-Whitney U test was utilised. These steps ensured rigorous data assessment.

6.4.6 Further experiments necessary

The data presented suggest further experiments are necessary to definitively prove some of the biological findings.

Variation of cell phenotype with prolonged culture suggests temporal change in monocytes. The use of maturation markers in this context would have been useful, and the quantification of CD68 expression in permeabilised cells, would give an indication of maturation to a more functionally phagocytic cell (Holness & Simmons 1993). The introduction of a further fluorochrome would enable multi-colour analysis in this regard, although with the caveat that membrane as well as surface expression would be notable for all molecules studied in this

manner. Such data would not necessarily correlate with the data already produced in this chapter.

Functional assays were not embarked upon but would be necessary to further corroborate surface molecule expression with leukocyte activation status. Particular focus upon migration, response to cytokine and chemokine influences and protease expression, would allow the relevance of the molecular changes described to be related to potential pathogenic mechanisms. In particular the alteration of monocyte migratory capacity through artificial matrices would be of interest. Gels made of extracellular matrix components would be used to assess alteration of monocyte migration with specific reference to matrix proteins that bind β2 integrins. Specific assays to explore the movement of lipid-laden macrophages through collagen and fibrinogen gels would illustrate whether β2 integrin mediated mobility changes are altered. Use of micro-porous culture wells would enable simple assessments of monocyte migratory capacity mimicking egress form the vascular wall. Such experiments could be further developed with the use of endothelial cell monolayers to model trans-endothelial migration. Phenotyping of molecules, in particular CD49d, needs to be performed in suspension culture as well as adhesion culture, before embarking on more functional assays.

Further related experiments that would be useful in extending this work are discussed in section 6.5.

6.5 Discussion

Macrophages adopt surface molecular repertoires specific to the inflammatory sites they enter (reviewed in Chapter 1 and section 6.1). Surface expression of Fc receptors, markers of differentiation and adhesion molecules vary between alveolar macrophages, (Taylor et al. 2000), intestinal macrophages (Smythies et al. 2005) and monocyte-derived Kupffer cells in the liver (Yamamoto et al. 1995). Although monocyte/macrophage phenotype has been explored immunohistochemically (van der Wal 1992), monocyte surface molecule expression in response to defined lipoproteins has not been fully catalogued *in vitro*.

Monocyte culture with $15dPGJ_2$ did not appear to cause marked alterations in surface phenotype and the limited sample number for these experiments makes alterations in cyPG mediated phenotypic switch difficult to assess. Clear changes were observed following monocyte exposure to differentially modified LDL, and are discussed in detail.

6.5.1 Pattern recognition receptor responses to LDL: CD14

CD14 links monocyte differentiation to function. CD14+/CD34 mononuclear subsets are a source of endothelial progenitor cells (Romagnani et al. 2005), and CD14+ peripheral blood

monocytes are capable of differentiating into monocyte-derived mesenchymal progenitors (Kuwana et al. 2003) highlighting the remarkable plasticity of CD14+ populations. Monocyte activation by LPS augments CD14 expression (section 6.1.2), inhibiting phenotypic switching from monocyte to DC (Palucka et al. 1999), a differentiation step usually associated with CD14 loss (Santin et al. 1999). CD14 ligation promotes subsequent macrophage activation in a NF-κB dependent fashion (Guha & Mackman 2001) increasing superoxide release (Landmann et al. 1995). CD14 is altered by microenvironment, being down-regulated in response to extrinsic cytokine stimuli including IL-4 (Lue et al. 1991) and IL-13 (Cosentino et al. 1995) (section 6.1.2). Conversely IL-6 promotes monocyte CD14 expression (Ikewaki & Inoko 1991).

There appeared to be a reduction in CD14 expression following n-LDL exposure over 8 days in adherent culture (section 6.2.7.1). All cells demonstrated ToPro3 exclusion, making reduced cell viability an unlikely explanation for n-LDL mediated CD14 reduction, which may instead reflect the heterogeneity of CD14 expression across individual cells and individual donors, visible on the data scatter plots presented. Cholesterol and lipoproteins have been previously shown to stabilise monocyte CD14 expression, (Esfahani et al. 1993) in contradistinction to the n-LDL-induced effect reported here. CD14 expression was preserved in ox-LDL supplemented adherent monocytes, suggesting that monocyte/macrophage phenotype is maintained despite an atherogenic environment. In models of leukocyte transmigration, cells remaining in the sub-endothelial space displayed intact CD14 expression (Randolph et al. 1998), in keeping with the maintenance of CD14 expression in the adherent LDL-exposed monocytes (section 6.2.7.1). Further phenotyping to assess maturation of monocytes to macrophages might have been performed using anti-CD68 (macrosialin) mAb (Carlsen et al. 2004). Immuno-histochemical studies have used CD68 to localise macrophage infiltration in *ex vivo* atherosclerotic lesions (Geng et al. 1995) making this a useful tool in further studies.

6.5.1.1 Pattern recognition receptor responses to LDL: CD36

No alteration in the monocyte surface expression of the scavenger receptor CD36 was noted (section 6.2.7.2). Enhanced CD36 expression in response to ox-LDL and PPARγ ligation has been demonstrated in THP-1 monocytic cells (Tontonoz et al. 1998), which do not display the same surface molecular profiles as primary human monocytes, and may not accurately reflect primary cell responses to environment (Fleit & Kobasiuk 1991). However, in earlier experiments primary peripheral blood monocytes did increase CD36 expression in response to ciglitazone, (chapter 3, section 3.2.15), suggesting that high affinity PPARγ ligation is

capable of promoting CD36 up-regulation. Ox-LDL was obtained from the same source as used by Tontonoz et al., making variability in oxidation status unlikely, and electrophoretic mobility data (Chapter 3 Appendix) confirmed consistent lipid oxidation across samples. The concentration of fatty acid-derived ligands including HODE may be too low in ox-LDL to reproduce CD36 upregulation in primary cells. Small changes in CD36 copy number may not be apparent in a small sample size from a heterogenous donor population, demanding a further expansion of sample numbers to demonstrate changes in molecular expression.

CD36 is an important route for lipid entry to macrophages (Febbraio et al. 2000). PPARγ-RXR regulation of liver X receptor (LXR) transcription in turn modulates scavenger receptor SR-BI and ABC-1 transporter levels, promoting HDL binding and cholesterol efflux, (Chawla et al. 2001b) homeostatically limiting monocyte lipid accumulation. A further feature of transcriptional regulation of cholesterol trafficking in monocytes is the LXR and RXR mediated down-regulation of CD36 which may explain limited changes seen during these phenotyping studies.

CD36 has been reported to play a role in macrophage recognition of apoptotic cells *via* thrombospondin ligation (Fadok et al. 1992). Common molecular profiles may explain the avid uptake of ox-LDL by macrophages (Chang et al. 1999). Ox-LDL derived phospholipid epitopes, including 1-palmitoyl 2-(5-oxovaleroyl) phosphatidylcholine (POVPC), and antibodies malondialdehyde-LDL may share moieties with apoptotic cells, potentially affecting the phagocytosis of effete cells (Chang et al. 1999). In view of the fact that monocyte-derived DCs may present phagocytosed debris to the immune system, (Albert et al. 1998) this prompted the examination of monocyte surface molecules involved in adaptive as well as immunity.

6.5.2 Adhesion molecules

6.5.2.1 β1 integrin stability

Monocyte β1 integrin expression appeared unchanged following exposure to LDL in both suspension and adherent culture, although a significant decrement in β1 integrin signal was noted following exposure to higher concentrations of 15dPGJ2 (section 6.2.8.1). Cells sampled for the latter assay displayed ToPro3 exclusion and thus reduced β1 expression could not be ascribed to low monocyte viability. However, the small number of samples makes these data difficult to interpret. Further studies would be needed to confirm these findings, and to provide a fuller phenotype for early LDL exposure in adherent monocytes.

Although functional studies were not performed the stable expression of β1 integrin is still suggestive that signalling *via* this integrin may be intact in ox-LDL exposed monocytes. Ligand-binding regions of integrin beta subunits contain a type A-domain similar to that found in the binding region of von Willebrand factor. This alpha/beta "Rossmann" fold contains a metal ion-dependent adhesion site (MIDAS) located on the upper surface. During receptor activation, the beta subunit A-domain undergoes tertiary structural changes. The mAb 12G10 recognizes a unique cation-regulated epitope on the beta-1 A-domain. Induction of this epitope reflects integrin activation status and thus competence for ligand recognition. The ability of $Mn^{(2+)}$ and $Mg^{(2+)}$ to stimulate 12G10 binding is removed by mutation of the MIDAS motif, proving that MIDAS is a $Mn^{(2+)}/Mg^{(2+)}$ binding site and that site occupancy induces A-domain conformational changes. The mechanism of beta subunit A-domain activation appears to be distinct from that of the A-domains found in some integrin alpha subunits. In summary, because the epitope recognised by 12G10 equates to an "activation" epitope in β1 integrins (Mould et al. 2002), the data presented suggest that β1 integrin adhesion and subsequent downstream signalling is unlikely to be altered early after monocyte exposure to Ox-LDL. LDL-exposed monocytes may still be capable of trans-endothelial migration *via* β1 integrin mediated interactions (Weber et al. 1996), a process as crucial in the monocyte efflux from sites of inflammation as it is in leukocyte recruitment (Bellingan et al. 2002).

6.5.2.2 Reduced CD49d expression

Reductions in CD49d were noted in response to ox-LDL in monocytes (section 6.2.8.2), although only adherent cells were assessed. Fluorescence signals were at the low end of the scale, and the decrement seen, although statistically significant, was small in absolute terms. Free iron supplements, a source of oxidative damage, increase expression of monocyte VLA-4 and VCAM-1 in ECs, promoting *in vitro* monocyte/EC binding (Kartikasari et al. 2004). The small reduction of CD49d induced by ox-LDL observed here contradicts this study, perhaps suggesting that oxidation-related effects are much less marked using ox-LDL than with free metal ions. Reduced CD49d expression, even if marginal, may have functional implications for lipid-laden macrophages. MMP-1 expression is regulated by α4β1 and α5β1 ligation of fibronectin, as well as of VCAM-1 and CD54/ICAM-1 (Matias-Roman et al. 2005). MMP-1 proteolysis of matrix proteins facilitates leukocyte movement, as well as activating other pro-MMPs, notably MMP-2 and MMP-13 (Seiki et al. 2003). Ox-LDL mediated down-regulation of CD49d may thus limit macrophage mobility, but may also limit matrix degradation, a possibility that is of direct relevance to vascular injury and repair.

Elevated macrophage secretion of MMP has been directly linked to atherosclerotic plaque rupture in apoE null mice (Gough et al. 2006), and in cultured macrophages exposed to ox-LDL (Xu et al. 1999). The influence of α4 integrin signalling in controlling MMP secretion in lipid-enriched micro-environments is of interest, and may offer insights into matrix influences upon plaque stability. The exploration of CD49d in protease secretion as well as migration assays is thus a further area of study.

The down-regulation in CD49d was not matched by CD49e (section 6.2.8.3). CD49d is more important in mediating weak interactions than CD49e, aiding focal adhesion complex formation influencing cell adhesion and migration through matrices. Conversely, CD49e may be able to salvage adhesion in the absence of CD49d (Kassner et al. 1995). In contrast to α5 integrins, α4 mediated binding to fibronectin can be influenced by β1 ligation, with exposure to the 12G10 mAb causing reduced α4β1 mediated actin polymerisation, cell spreading and motility (Humphries et al. 2005). These studies suggest that reduced α4 expression in adherent cells may reduce mobility, but the influence of CD49e expression needs to be considered, and monocyte migration studies through defined matrices following blockade of CD49e would answer this.

The signalling cascades that follow α4 ligation present a further focus for study. α4 integrin-mediated cell migration is regulated by protein kinase A phosphorylation of the α4 cytoplasmic tail (Chan et al. 1992). Paxillin binding to the α4 integrin tail is essential for cell movement, but is inhibited by phosphorylation. The effects of modified LDL upon α4 integrin tails and the downstream signalling changes that this might invoke have not been explored, but might provide additional information relating to altered macrophage mobility within atherosclerotic plaques.

6.5.2.3 Monocyte CD11a expression

Despite suggestions from gene array data, (Chapter 5), no statistically significant changes in CD11a expression were apparent during suspension culture following LDL exposure (section 6.2.8.4). The early reduction in CD11a expression relative to control in adherent culture, although statistically significant, did not appear marked; it is questionable whether this would result in reduced CD11a-mediated responses. Surface CD11a thus appears to be stable (Bretscher 1992), when using the same adherence culture conditions for gene array preparation, and does not reflect the reduction in transcription noted in array and PCR work (Chapter 5). This is of interest as recent data has suggested that CD11a expression may be downregulated by linoleic acid (Rinker et al. 2004), in keeping with the transcriptional data

presented. Other modes of assessing CD11a during dynamic migration or mobility assays were considered in Chapter 5.

6.5.2.4 Reduced CD11b expression

CD11b appeared stable in early suspension culture with ox-LDL, but was reduced in prolonged adherent culture. Thus, although the phenotype analysis performed on monocytes in suspension culture was incomplete, with only 24hr data available, the reduction in CD11b expression appeared related to monocyte maturation and adhesion status, as well as oxidation status of LDL supplements (section 6.2.8.5). No initial up-regulation in suspension culture was obvious following ox-LDL exposure, in contrast to clinical data (Weber et al. 1997) and histological studies in primates (Strawn et al. 2000) showing elevated leukocyte CD11b expression in hyperlipidaemia. However, *in vitro* data using THP-1 cells suggests that CD11b remains stable following ox-LDL exposure, and is only up-regulated in response to CC chemokines (Han et al. 2003). Other studies have suggested that circulating leukocytes do not display basal increases in CD11b expression in hyperlipidaemic states alone (Couffinhal et al. 1993), but CD11b may be rapidly up-regulated during clinical vascular events including peripheral arterial disease (Mazzone et al. 1997). Evidence to support the observed down-regulation of CD11b in adherent monocytes comes from studies in rabbit models of atherosclerosis. Animals on a high cholesterol diet were subject to aortic barotrauma, and monocytes were then sampled from lesions. Lower levels of CD11b were noted in monocytes derived from lesions in hyperlipidaemic animals, with the suggestion that this compromised macrophage emigration from plaques (Gray & Shankar 1995). However, this study attempted to compare lesional macrophages with circulating monocytes, and did not clearly distinguish the role of responses to mechanical injury from those of responses to dyslipidaemia. Electron microscopy work has shown that macrophages are capable of leaving atherosclerotic plaque and re-entering the vessel lumen (Gerrity 1981b). Mac-1 deficient mice confirm the role of β2 integrins in aiding the exit of macrophages from inflammatory sites (Cao et al. 2005). The differential responses to ox-LDL may suggest that mature adherent monocytes selectively down-regulate CD11b expression producing a poorly motile or sessile resident macrophage within atherosclerotic plaque.

6.5.2.5 Reduced CD11c expression

CD11c expression was also reduced following ox-LDL exposure in adherent culture alone (section 6.2.8.6). In conjunction with preserved CD14 expression this supports a mature monocyte phenotype following LDL exposure, in distinction from CD11c+ dendritic cells.

Immuno-histochemical studies of atherosclerotic plaque have shown that some CD68+ monocyte derived cells express high levels of the DC marker CD1a, (Spanbroek et al. 2003), and CD1+ve cells harvested from atherosclerotic plaque are capable of presenting lipid antigens to T cells *in vitro* (Melian et al. 1999). Adoption of a DC surface phenotype has been observed in monocytes leaving atherosclerotic plaques (Llodra et al. 2004), complementing phenotypic data from *in vitro* models of transmigration (Randolph et al. 1998). Monocytic cells that successfully re-traverse the endothelium have marked increases in both CD11c and CD11b, along with a decrease in CD14 expression (Muller & Randolph 1999). A co-ordinated down-regulation of both CD11c and CD11b expression may thus limit macrophage mobility within the vessel wall, as well as reducing reverse transmigration. Further study of lipid effects upon DC antigen presentation and mobility would be useful. More attention should perhaps be paid to lipid-laden immotile CD11c-low monocytes, in assessing their immune competence and migratory capacity.

6.5.2.6 CD61 (β3) integrin expression

Changes in monocyte surface CD61 (β3/GPIIIa) expression were noted to be statistically significant following early n-LDL and late ox-LDL exposure, although this was only assessed in adherent culture. The alterations were all small in terms of total fluorescence, with signals at the lower end of the cytometer dynamic range. The biological significance of such changes in debatable, as the actual alteration in surface copy number is unlikely to produce a functional change in adhesion. A fuller phenotype to assess expression during other culture conditions might help ascertain whether these alterations are relevant to monocyte function and the use of alternative mAb and fluorochromes might assist qunatification. CD61 plays an established but distinct role in platelet aggregation, clearly relevant to complex atherothrombotic events. CD61-null murine studies (section 6.1.6.2) have suggested that CD61 tonically inhibits atherogenesis by limiting CD36 and CD40/CD40L mediated signalling (Weng et al. 2003). However, it is also likely that αvβ3 integrin mediated signalling contributes to VSMC migration and growth (Hoshiga et al. 1995), potentially promoting arterial wall remodelling and reducing vessel calibre. It is likely that CD61 mediates differing effects particular to leukocyte, VSMC or platelet, a hypothesis strengthened by studies of polymorphisms of the PlA gene. Increased thrombotic events appear associated with platelet PlA/A1 polymorphisms, whereas progressive fibrotic atheroma is associated with VSMC-expressed PlA/A2 (Mikkelsson et al. 2001). Even if monocyte surface CD61 expression is unaltered by modified LDL, αvβ3 plays an important role in phagocytic clearance (Savill et al. 1990) relevant to atherosclerosis, and CD61 is

important in vascular injury during atherothrombotic complications and vascular remodelling. The exploration of CD61 responses to modified LDL and resultant effects upon phagocytosis would be an obvious target for further work.

6.5.2.7 Changes in CD54 (ICAM-1) expression

Monocyte CD54 expression appeared dependent upon lipid oxidation, adhesion status and maturation. Although monocytes in suspension culture were only assessed at 24hrs, the up-regulation of CD54 following ox-LDL exposure contrasted with the stable expression noted following early adherent culture, and the marked down-regulation that followed protracted adherent culture with ox-LDL (section 6.2.8.8). Suspension culture data may reflect the responses of uncommitted monocytes, although it is unclear as to whether they would be exposed to this level of ox-LDL *in vivo*, and studies of monocytes in suspension exposed to a range of ox-LDL concentrations would be useful in this regard. Ox-LDL mediated up-regulation of CD54 in non-adherent human primary monocytes suggests an additional pro-adherent effect of ox-LDL upon monocytes, and has not been previously reported. However, changes in CD54 responses to ox-LDL have been noted in U937 monocytic cells with a peak in CD54 transcription after 12hrs exposure (Yang & Rui 2003). CD54 mRNA levels returned to baseline after 48hrs, suggesting a dynamic response to ox-LDL. Although resident macrophages in coronary atheroma have been shown to express CD54, (Davies et al. 1993) these data were neither quantified, nor correlated to the stage of the individual plaque. CD54 is essential for T cell priming, enhancing responses to B7 molecules (Damle et al. 1992), and supports Class II MHC-mediated antigen presentation to T cells, (Altmann et al. 1989). Studies of monocyte responses to ox-LDL by atheroma biologists have focused upon adhesion to ECs or VSMCs (Thorne et al. 1996), without exploring monocyte surface adhesion molecule expression. The data presented are of interest in highlighting CD54 changes in response to ox-LDL, and demand further investigation from both adhesion and adaptive immune perspectives.

6.5.3 Monocyte surface molecules mediating adaptive immune function

6.5.3.1 Immunoglobulin receptors

The reported uptake of ox-LDL incorporated into immune complexes *via* a CD64(FcγRI) mediated route (Huang et al. 1999b), suggests that CD64 expression may be an important route for monocytes to accumulation lipid. However, no significant changes in CD64 expression were seen during adherent cell exposure to ox-LDL. Although an early reduction in ox-LDL treated cells compared with n-LDL treated cells was noted during total

fluorescence quantification, this was not significant when adjusted for control fluorescence, (section 6.2.9.1). Immunoglobulin binding studies would clarify whether the stable expression of CD64 *in vitro* in lipid-rich monocytes reflects functional competence. An alternate mechanism may link atherosclerosis with CD64 mediated inflammatory mechanisms. Pentraxin proteins including serum amyloid protein and C-reactive protein (CRP) promote opsonisation and ingestion of apoptotic cells by phagocytes *via* CD64 (Mold, Baca, & Du Clos 2002), and evidence demonstrates that raised CRP levels are associated with adverse clinical outcomes in arterial disease (Ridker, Stampfer, & Rifai 2001). Stable CD64 expression in lipid-laden monocytes may thus be augmented *in vivo* by circulating CRP, promoting phagocytosis in an attempt to clear cell debris at sites of vascular inflammation. The presence of CD64 in human atheroma has been demonstrated immuno-histochemically (Ratcliffe, Kennedy, & Morganelli 2001). Determination of relative levels of CD64 expression within plaque would allow the CD64 levels diminished as plaque progression occurred. Additional insight might be gained from animal models of atherosclerosis, to ascertain if CD64 levels were associated with reparative monocyte responses that parallel those seen in arthritis and bacterial infection (Ioan-Facsinay et al. 2002).

It is perhaps surprising that CD16/FcγRIIIa expression was unchanged in LDL-exposed monocytes (section 6.2.9.2). CD16/FcγRIIIb shedding is well documented during granulocyte activation (Huizinga et al. 1988). Shedding of CD16/FcγRIIIa has been reported in inflammatory disease with elevated plasma levels of soluble CD16/FcγRIIIa found in active rheumatoid arthritis (Masuda et al. 2003). It is possible that concomitant cytokine input might unmask changes in CD16/FcγRIIIa expression. TGF-β1 can reduce γ-chain expression, lowering monocyte surface CD64 and CD16, and reducing FcγR mediated production of MCP-1 (Tridandapani et al. 2003). Examination of parallel LDL-mediated changes in monocyte FcR expression and function would thus require *in vitro* culture with individual cytokines and co-culture with lymphocytes, or lymphocyte-conditioned media. This needs further exploration using blocking antibodies in combination with aggregated LDL particles. Finally, individual FcR influences upon macrophage behaviour in hyperlipidaemic states could be elucidated by the use of gene deletion studies against an apoE background, demonstrating whether defective binding to immune complexes containing LDL altered atherosclerotic plaque load or morphology *in vivo*.

CD32/FcγRII is important in binding immune complexes. Neutrophil binding to immune complexes is mediated by CD32, augmenting phagocytosis of opsonised apoptotic cells (Hart, Alexander, & Dransfield 2004). Although CD32 plays a role in the binding of LDL to

leukocytes, it was not explored because of time constraints. However, further assessment of Fc receptor binding of aggregated LDL particles would require the assessment of CD32 expression and function.

6.5.3.2 Class II MHC molecule expression

An early alteration of monocyte Class II expression relative to control was seen in adherent but not suspension culture in the presence of ox-LDL (section 6.2.9.3). Total fluorescence was not statistically altered, and a reduction in Class II expression was not apparent after prolonged adherent culture. Such data are difficult to interpret in the context of their biological significance, as changes in absolute fluorescence on histograms appeared minor. Moreover, although it is possible that molecular copy number, and indeed functional competence of Class II proteins, might be reduced, this would require confirmation using formal assays of antigen presentation. In the Rag 2/apoE null model of atherosclerosis, atherogenesis progressed despite the absence of T cells but with reduced lesional macrophage Class II expression (Daugherty et al. 1997), suggesting that T cell input is important in modifying plaque macrophage phenotype *in vivo*. Class II MHC null mice fed a pro-atherogenic diet, also show normal progression of atherosclerosis, (Fyfe, Qiao, & Lusis 1994), but no formal studies of macrophage function were performed with these animals. Additional information about lipid effects upon Class II function and monocyte antigen processing capability might be gained by examining antigen presentation in mixed leukocyte reactions (Krieger et al. 1988). Further studies using defined apoprotein-derived peptide epitopes would help assess whether antigen presentation is altered in lipid laden monocytes, and whether exposure to ox-LDL alters these responses. The expression of Class II molecules at the macrophage surface is promoted by T lymphocyte cytokine input, particularly IFN-γ (Steeg et al. 1982) and IL-4 (Stuart, Zlotnik, & Woodward 1988). The influence of T lymphocyte cross-talk upon lipid-laden macrophages thus merits further exploration, and the behaviour of macrophages in co-culture would be of particular interest.

6.5.3.3 Co-stimulatory molecules and other immune adaptors

No alteration of CD86 (B7.2) expression by LDL was noted during monocyte phenotyping in suspension or adherent culture (section 6.2.9.4). The ability of lipid laden VSMCs ECs and macrophages to initiate B7 up-regulation signal in B cells *via* CD40 has been demonstrated (Buono et al. 2004), although no functional data on macrophage B7 responses to lipid loading have been published to date.

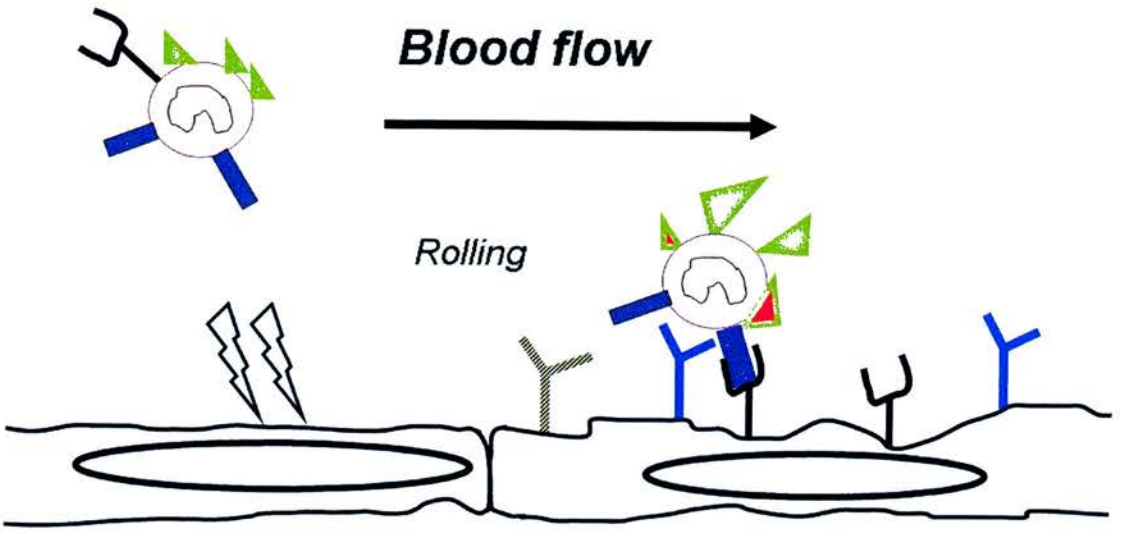
Both CD80 (B7.1) and CD86 (B7.2) molecules bind to CTLA-4 and CD28 on T helper (Th) cell surfaces promoting T cell activation (Linsley et al. 1994) (see section 6.1.9.2), driving T

cells along a Th1 or Th2 phenotypic path. Th1 cells promote a classical path of macrophage activation enhancing the production of pro-inflammatory cytokines including IFNγ aiding cellular immune responses to mycobacterium and HIV. Th2 lymphocytes assists humoral immune responses and are associated with some anti-inflammatory cytokine influences upon macrophages, with the production of IL-4, IL-10 and IL-13 (reviewed by Gordon 2003). T cell ligation of CD86 molecules favours a switch to a Th2 phenotype, enhancing IL-4 production (Ranger et al. 1996). CD80 ligation promotes Th1 production of IL-2 and IFNγ activating macrophages (Kuchroo et al. 1995). The resulting pro-inflammatory state may be relevant to atherosclerosis, as isolated monocytes from patients suffering acute coronary syndromes exhibit elevated CD80 expression following *ex vivo* LPS stimulation (Methe et al. 2005).

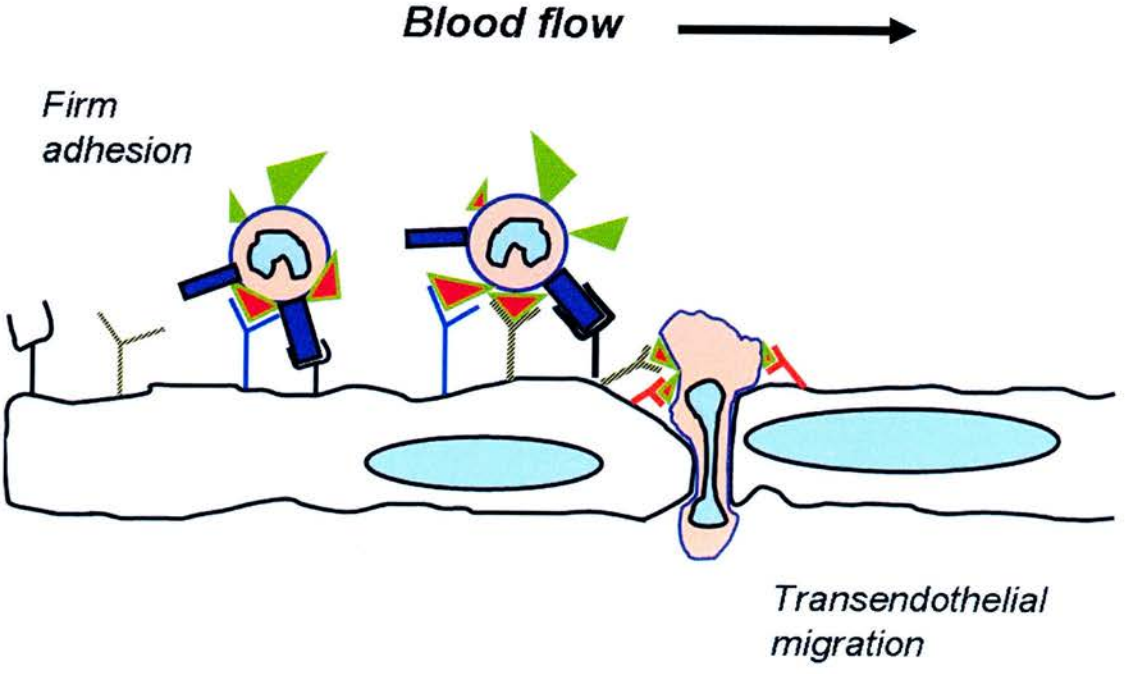
Although gene deletion studies show that individual B7 molecule changes may be insufficient to alter T cell responses (Borriello et al. 1997), it would be useful to assess CD80 expression before commenting on LDL-mediated effects upon macrophage co-stimulatory molecules. If CD80 molecular expression is also maintained functional assays would need to be undertaken to confirm that lipid-laden monocytes are capable of B7 mediated signalling. This would be possible using mixed leukocyte reactions (MLR) and mAb blocking strategies to assess cytokine responses to B7 ligation. The possibility that *in vivo* B7 molecular signalling might be altered in hyperlipidaemic and pro-inflammatory states thus deserves further investigation.

6.6 Overview

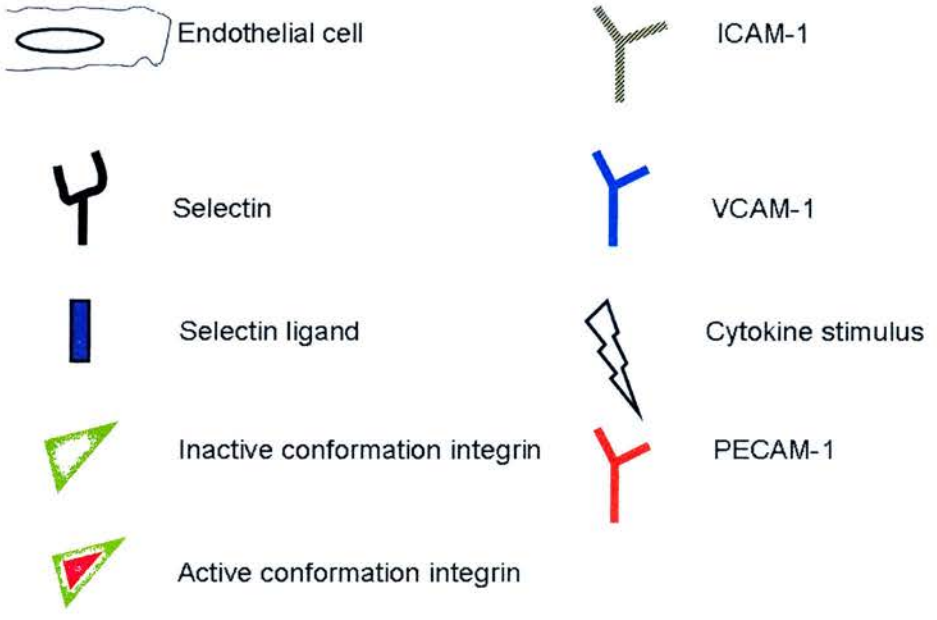
Monocyte phenotype is altered by modified LDL in an adhesion and maturation dependent fashion. No effects upon pattern recognition receptors or monocyte immuno-regulatory molecules were noted, but significant changes in expression of specific adhesion molecules were seen following exposure to ox-LDL. The implications these findings may have for monocyte migration and mobility within atherosclerotic plaque, along with a general consideration of monocyte phenotype and immune function in atherosclerosis will be discussed further in Chapter 7.


Blood flow
Rolling

a)


Blood flow
Firm
adhesion
Transendothelial
migration

b)



c) Key to figure 6.1

Figure 6-1 Multi-step paradigm of cell recruitment

Unactivated intravascular leukocytes and endothelial cells (Figure 6.1a) do not interact. Selectin-binding ligands are constitutively expressed on leukocytes. Quiescent endothelial cells do not express corresponding selectins. Endothelial injury and cell activation upregulates selectin expression, initiating leukocyte-rolling.

Leukocyte activation enhances leukocyte integrin binding to Ig-supergene family glycoproteins including ICAM-1 and VCAM-1, permitting firm adhesion. Transendothelial migration (Figure 6.1b) is then facilitated by other Ig-supergene family members including endothelial PECAM-1.

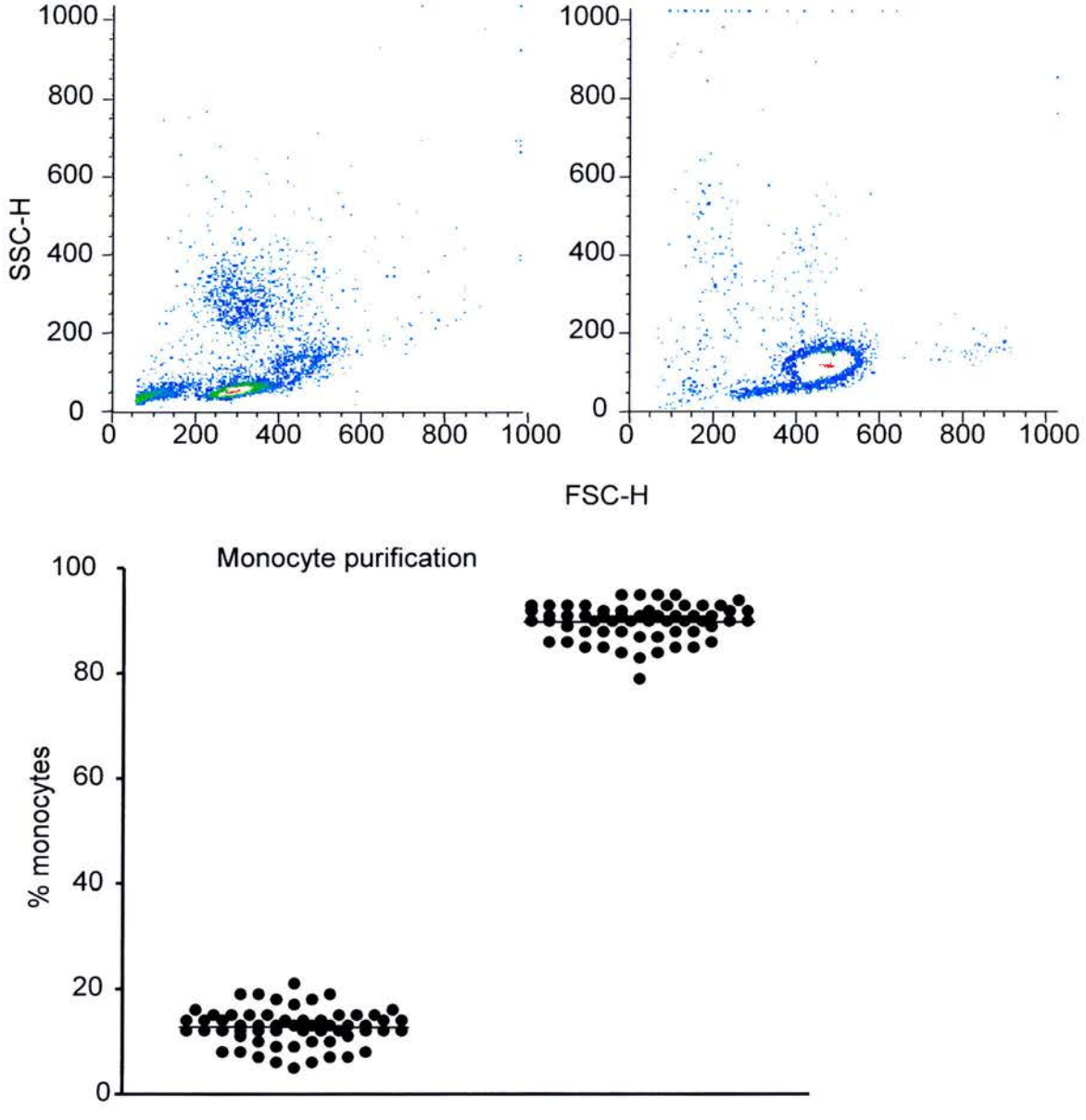


Figure 6-2 Monocyte isolation

Density gradient centrifugation produces mononuclear fractions (upper left panel). Use of immunomagnetic separation techniques enables removal of contaminating lymphocytes, NK cells, megakaryocytes, platelets and residual granulocytes upper right panel. Percentage monocytes are shown for 60 leukocyte preparations in the lower panel.

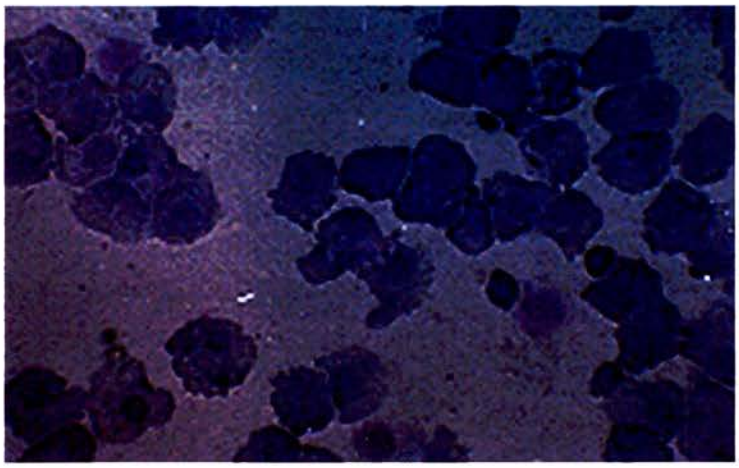


Figure 6-3 Light microscopy of control monocytes

Monocytes cultured in adherent serum-replete conditions with control medium (shown above) maintain normal macrophage ultra-structure, with preserved cytoplasmic appearances.

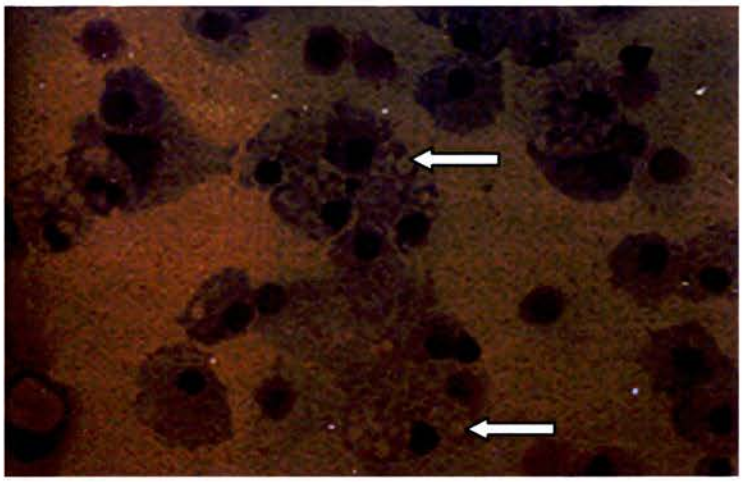


Figure 6-4 Light microscopy of ox-LDL supplemented monocytes

Monocytes exposed to oxidised LDL in adherent serum-replete culture increase lipid ingestion and adopt a lipid-laden vacuolated phenotype (arrows).
Fixation of cytocentrifuge preparations with alcohol dissolves and removes lipids, a technique used in differential staining. Lipid inclusions in mature macrophages thus appear as marked vacuoles.

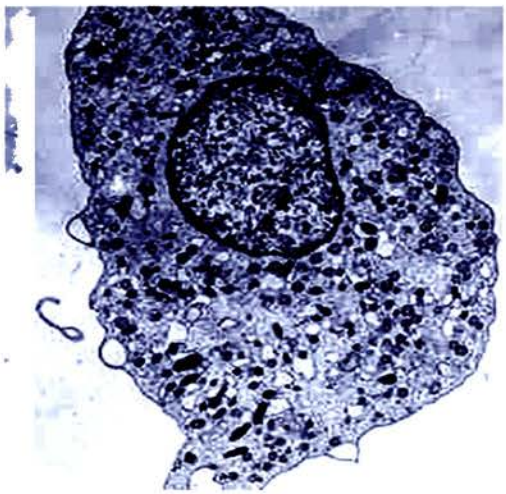


Figure 6-5 Transmission electron micrograph: control monocyte 7 days

Monocytes in serum-replete suspension culture maintain a cytoplasmic appearance consistent with maturation towards a macrophage phenotype (x10,000).

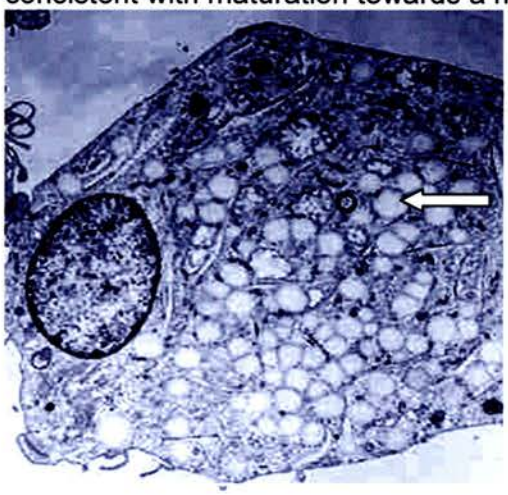


Figure 6-6 Transmission electron micrograph: monocyte cultured in ox-LDL 7 days

Monocytes cultured in serum-replete suspension conditions supplemented with 50μg/ml ox-LDL show marked increases in intra-cytoplasmic lipid inclusions (arrow). Although intra-cytoplasmic vacuolation is a mark of macrophage maturation, this degree of intracellular inclusion is higher than that seen in control cells (x10,000).

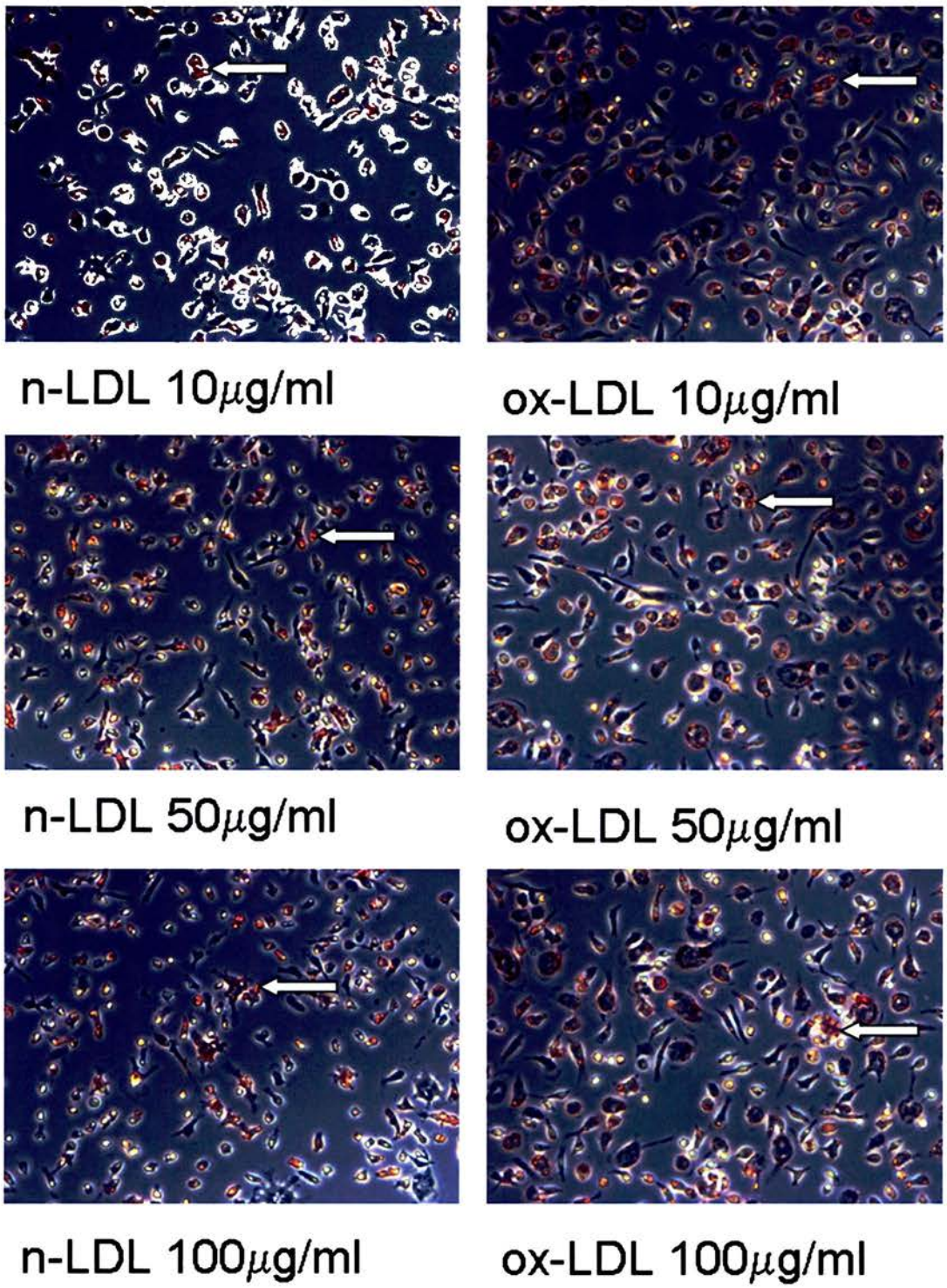


Figure 6-7 Oil Red O uptake: adherent monocytes, 24 hrs, LDL supplements

Adherent macrophages cultured in medium supplemented with increasing concentrations of n-LDL and ox-LDL were labelled with Oil Red O. Uptake of ORO is evident at early time points (arrows) consistent with the presence of intracellular cholesterol ester and triglycerides (x10 magnification).

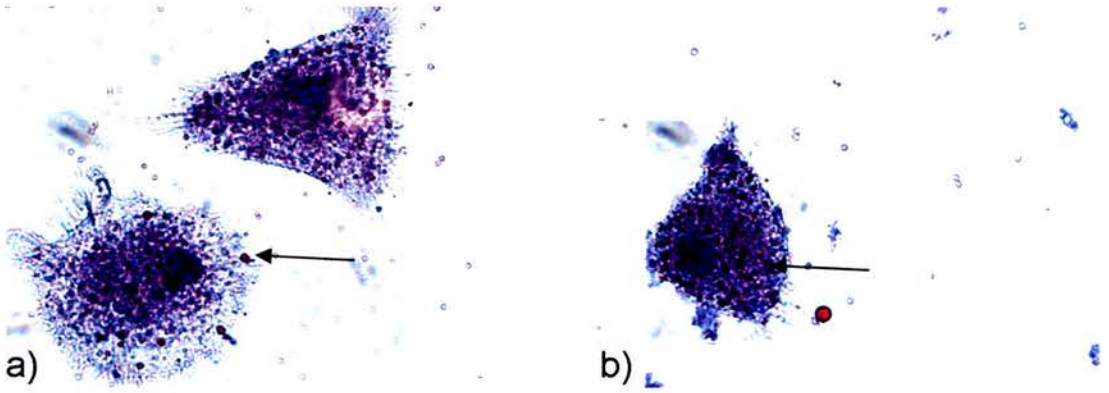


Figure 6-8 Control monocytes, adherent culture, 8 days, Oil Red O labelling
Low levels of uptake of oil red O (arrows) visible in panels a) and b) are seen following prolonged culture in serum-replete media (x100, oil immersion).

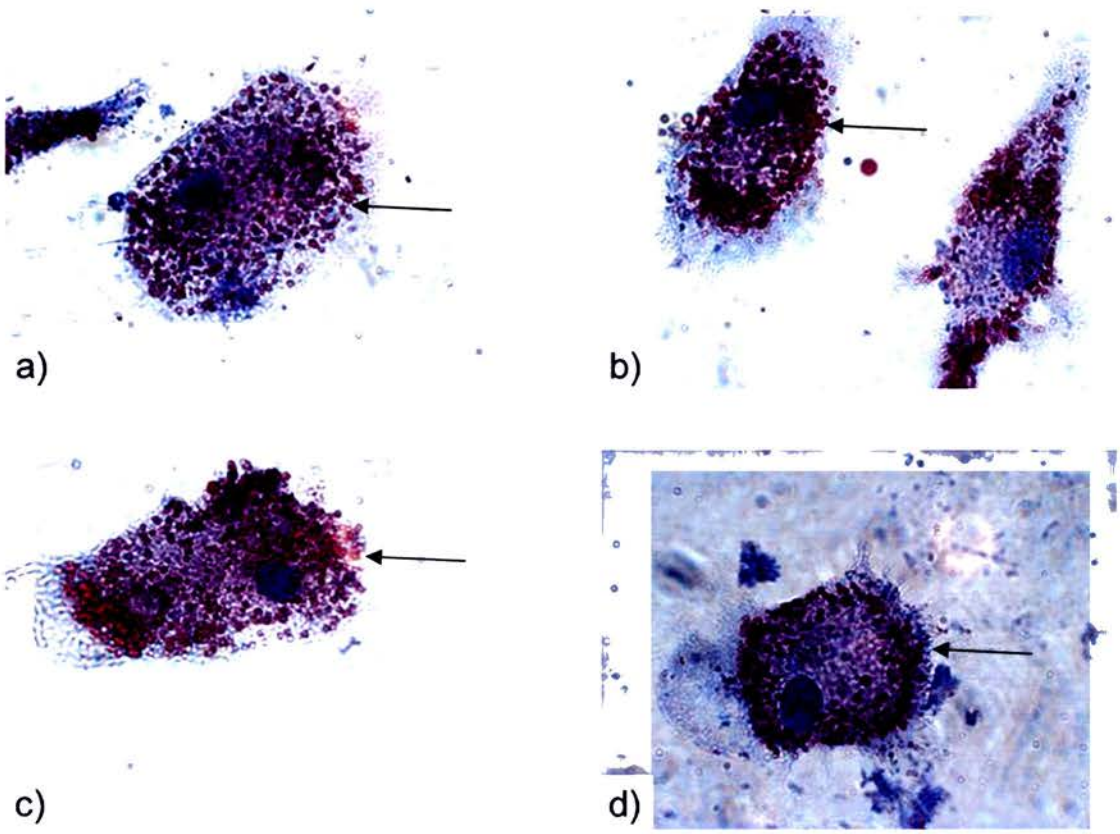


Figure 6-9 Monocyte oil red O uptake, adherent culture, n-LDL supplements
Monocytes supplemented with n-LDL at 10μg/ml (panel a), 50μg/ml (panel b) and 100μg/ml (panels c and d) show increased levels of oil red O labelling (arrows) at 8 days in adherent culture (x100, oil immersion).

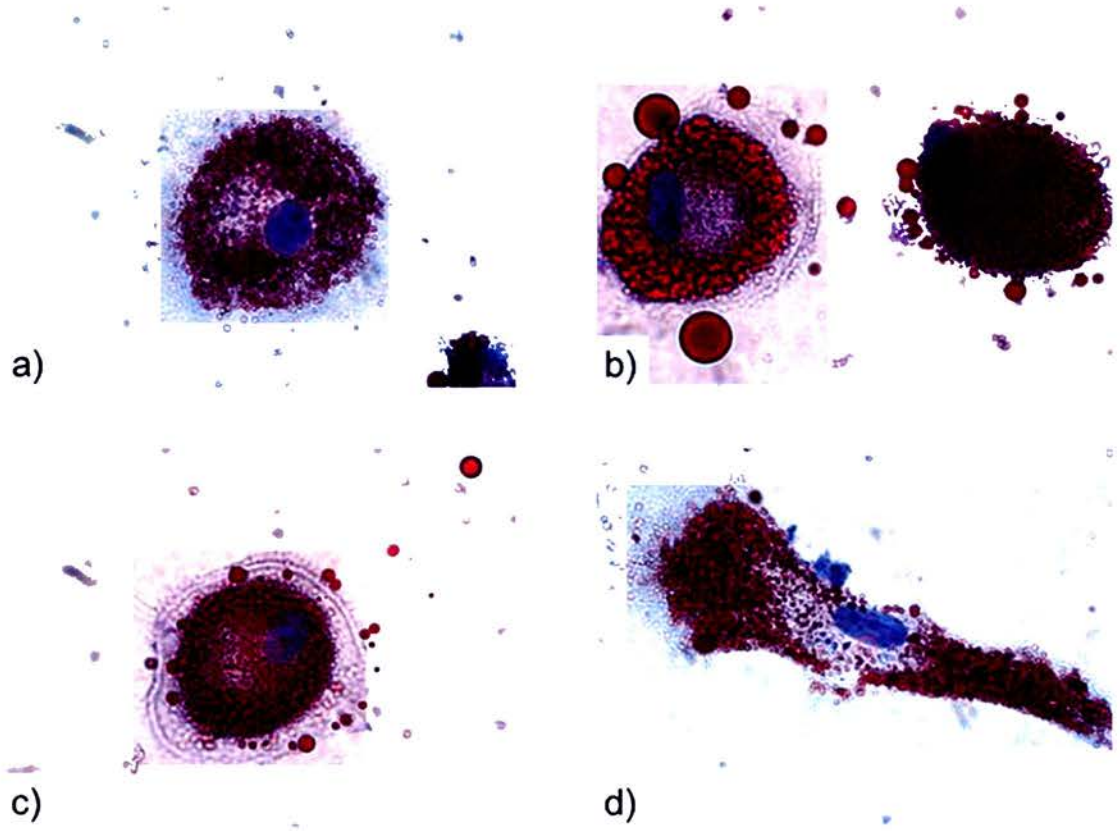


Figure 6-10 Monocyte oil red O uptake, adherent culture, 8 days, ox-LDL supplements

Monocytes cultured in ox-LDL at 10μg/ml (panel a), 50μg/ml (panel b) and 100μg/ml (panels c and d) show increased levels of intracellular oil red O at 8 days in adherent culture. Some free oil red O is visible in micrographs 6.10b and c, (x100 oil immersion).

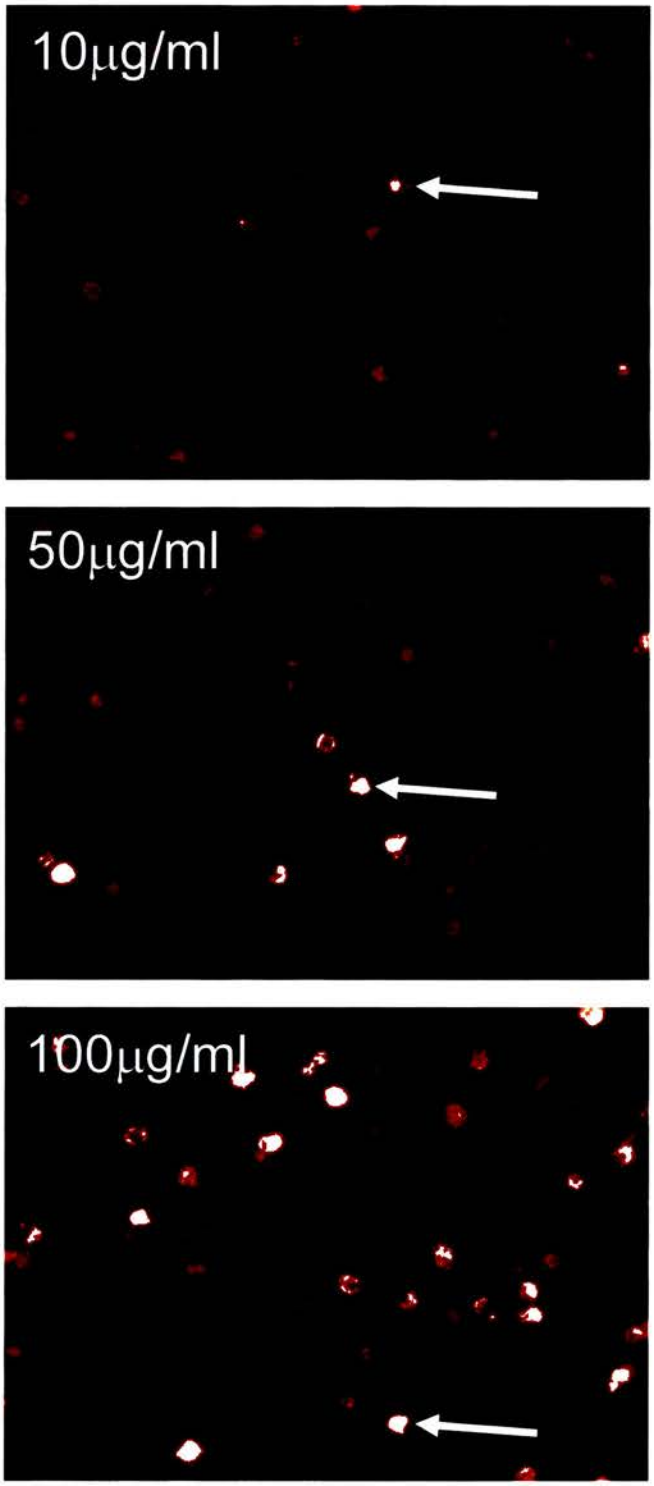


Figure 6-11 Monocyte ox-LDL uptake, 24hrs

Monocytes cultured with 10μg/ml (a) 50μg/ml (b) or 100μg/ml (c) Di-I labelled ox-LDL showed increasing levels of LDL uptake (arrows) after 24hrs in adherent culture, (x32, fluorescent microscopy).

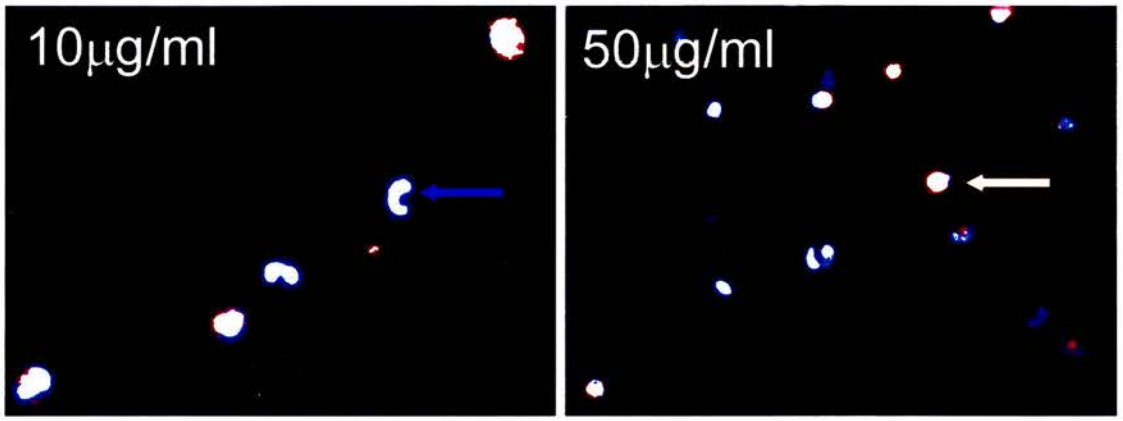


Figure 6-12 Monocyte Di-I labelled ox-LDL uptake, 24hrs, adherent culture.
Nuclei are labelled blue with Hoechst 33428 (blue arrow) demonstrating monocyte viability in adherent culture, with concomitant Di-I labelled ox-LDL uptake (orange arrow), at 10μg/ml and 50μg/ml

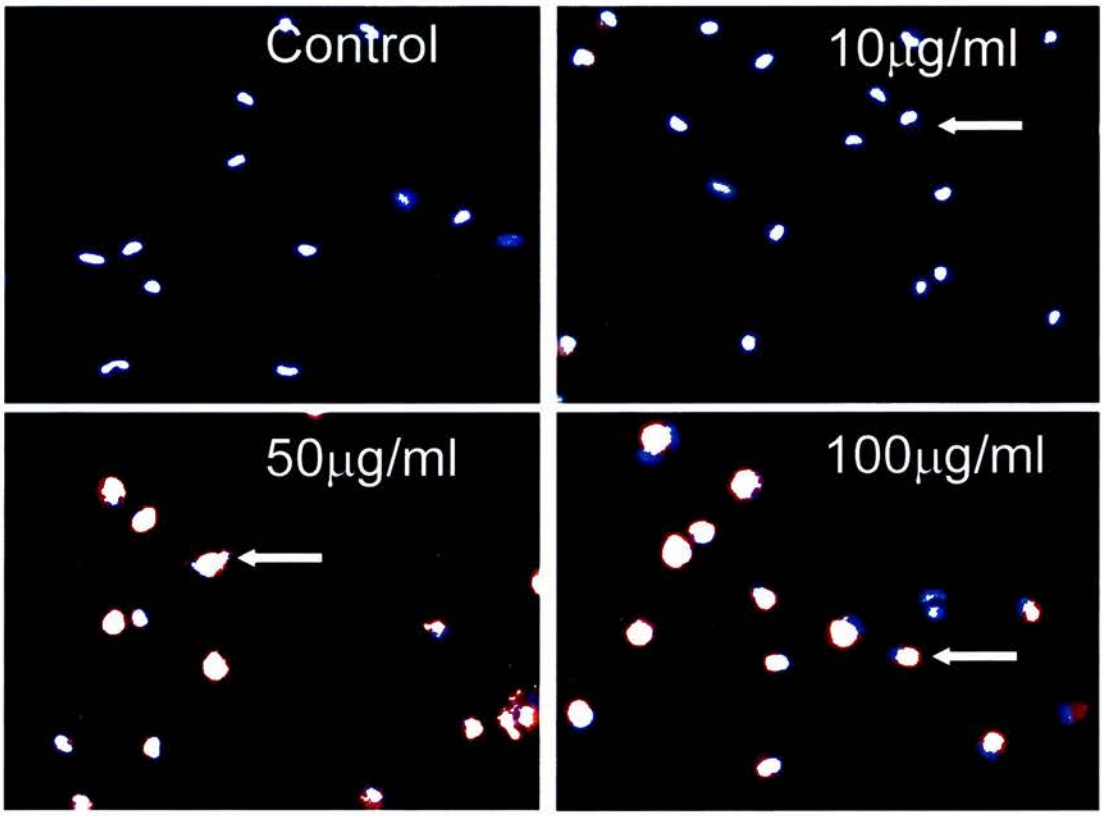


Figure 6-13 Monocyte Di-I labelled ox-LDL uptake, 48hrs adherent culture.
Monocytes cultured in ox-LDL for 48 hrs show marked uptake of Di-I labelled ox-LDL (arrows). Viability is confirmed by nuclear counter-labelling with Hoechst 33428.

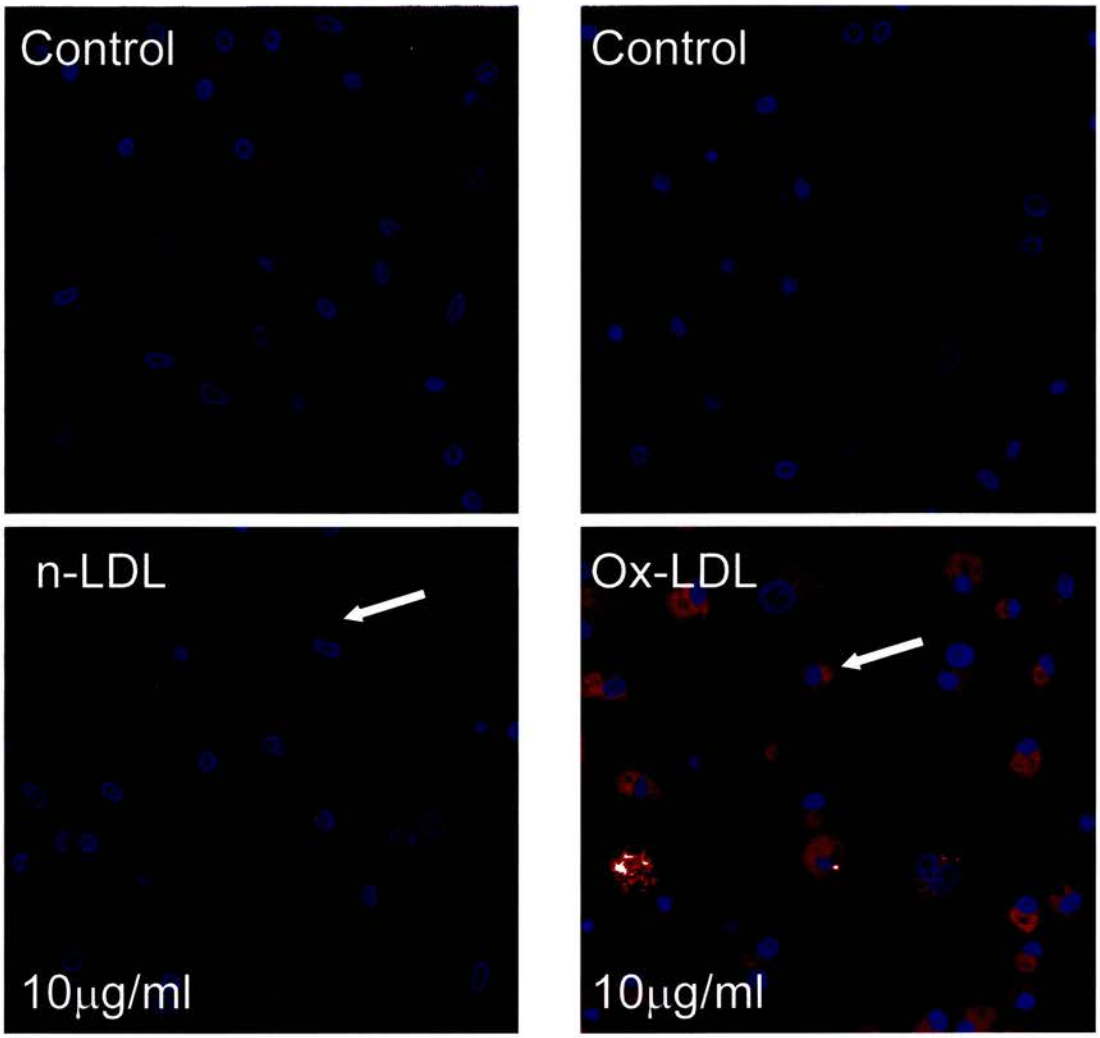


Figure 6-14 Monocyte LDL uptake, 8 days adherent culture, low concentration LDL

Contemporaneously cultured human monocytes were exposed to control media or Di-I labelled n-LDL (left-hand panels) or ox-LDL (right-hand panels) at increasing concentrations. Cells were fixed after 8 days adherent culture, and nuclei labelled with ToPro3.
Cells were imaged with a Zeiss confocal microscope using a x40 objective under oil immersion. Nuclei appear blue, and Di-I LDL appears red (arrows).

Nuclear labelling alone is seen in control samples.
Evidence of intracellular lipid uptake is apparent in both n-LDL and ox-LDL exposed monocytes at low concentrations, but n-LDL uptake appears much less avid than ox-LDL uptake.

Un-conjugated Di-I labels cell membranes alone, and is not shown.

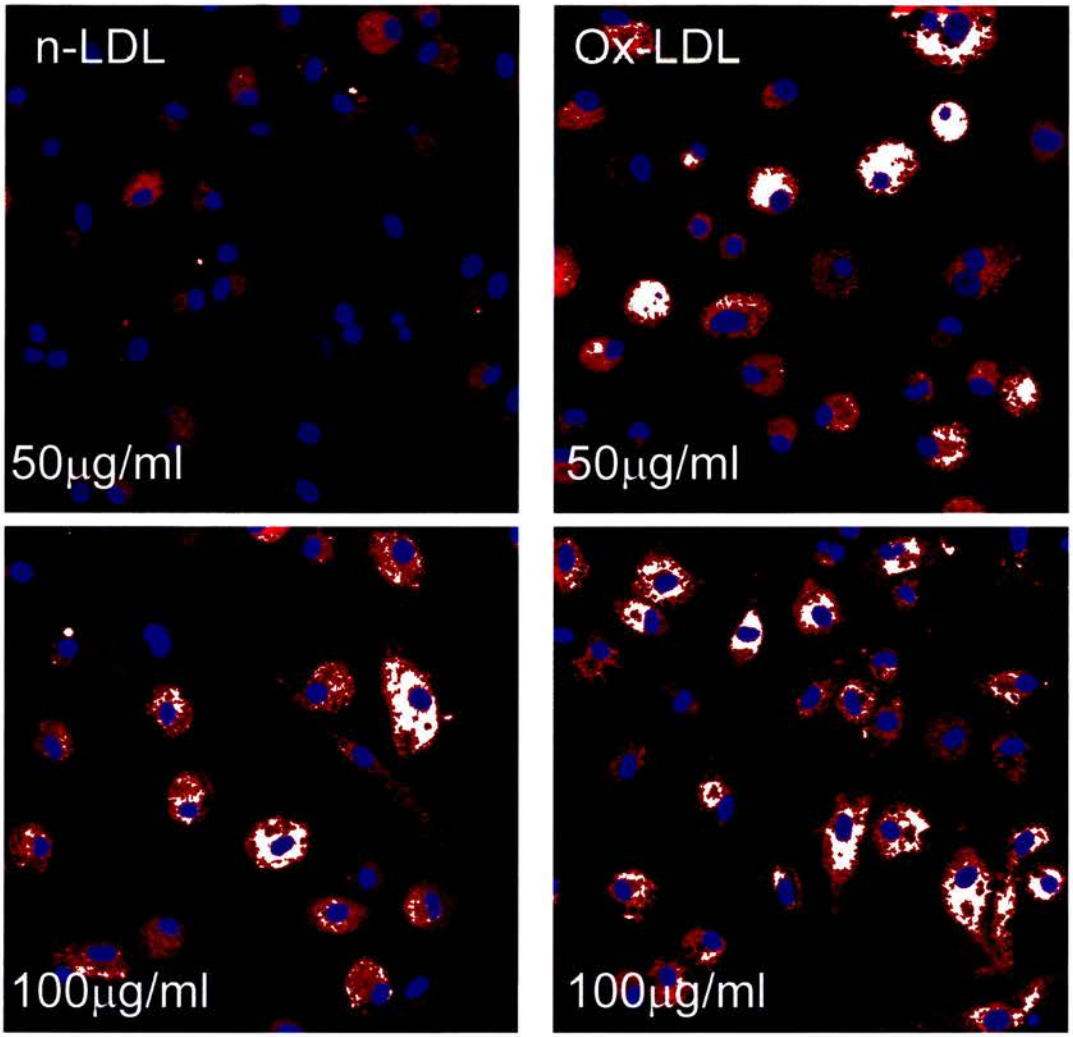


Figure 6-15 Monocyte LDL uptake, 8 days adherent culture, medium and high concentration LDL

Monocytes were prepared contemporaneously, as for Figure 6.14.

Monocytes were exposed to Di-I labelled n-LDL (left-hand panels), or ox-LDL (right-hand panels) at 50μg/ml and 100μg/ml.

Evidence of intracellular lipid uptake is visible in all LDL exposed monocytes.

An increase in red fluorescence proportional to the concentration of LDL exposure is apparent.
n-LDL appears less avidly taken up than ox-LDL at all concentrations used, although the highest concentration of n-LDL is more comparable to the corresponding ox-LDL concentration.

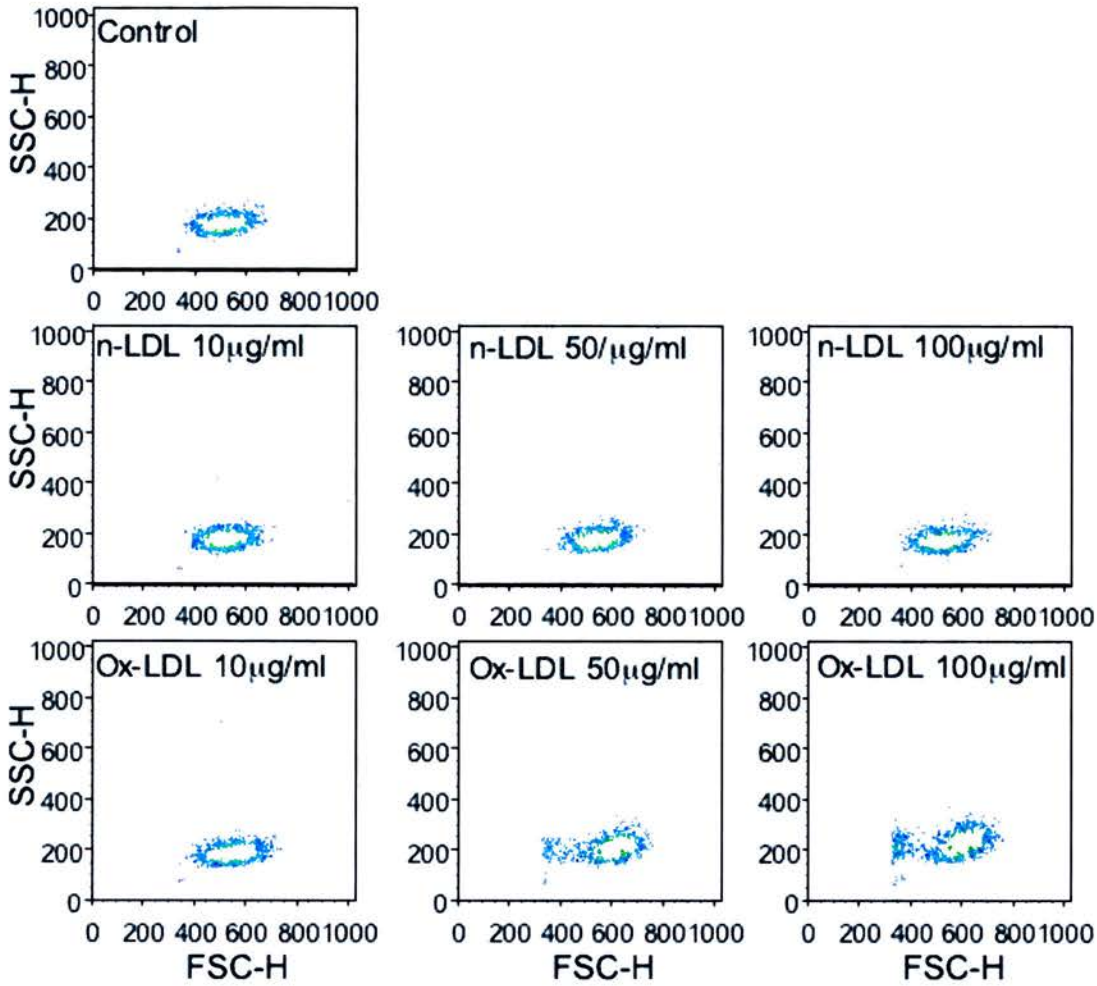


Figure 6-16 Scatter characteristics of LDL supplemented monocytes, early suspension culture

Monocytes cultured in suspension for 24 hours in serum-replete control media or media supplemented with increasing concentrations of native or oxidised LDL. Side scatter and forward scatter properties are altered at early time points after exposure to ox-LDL at 50μg/ml and 100μg/ml.

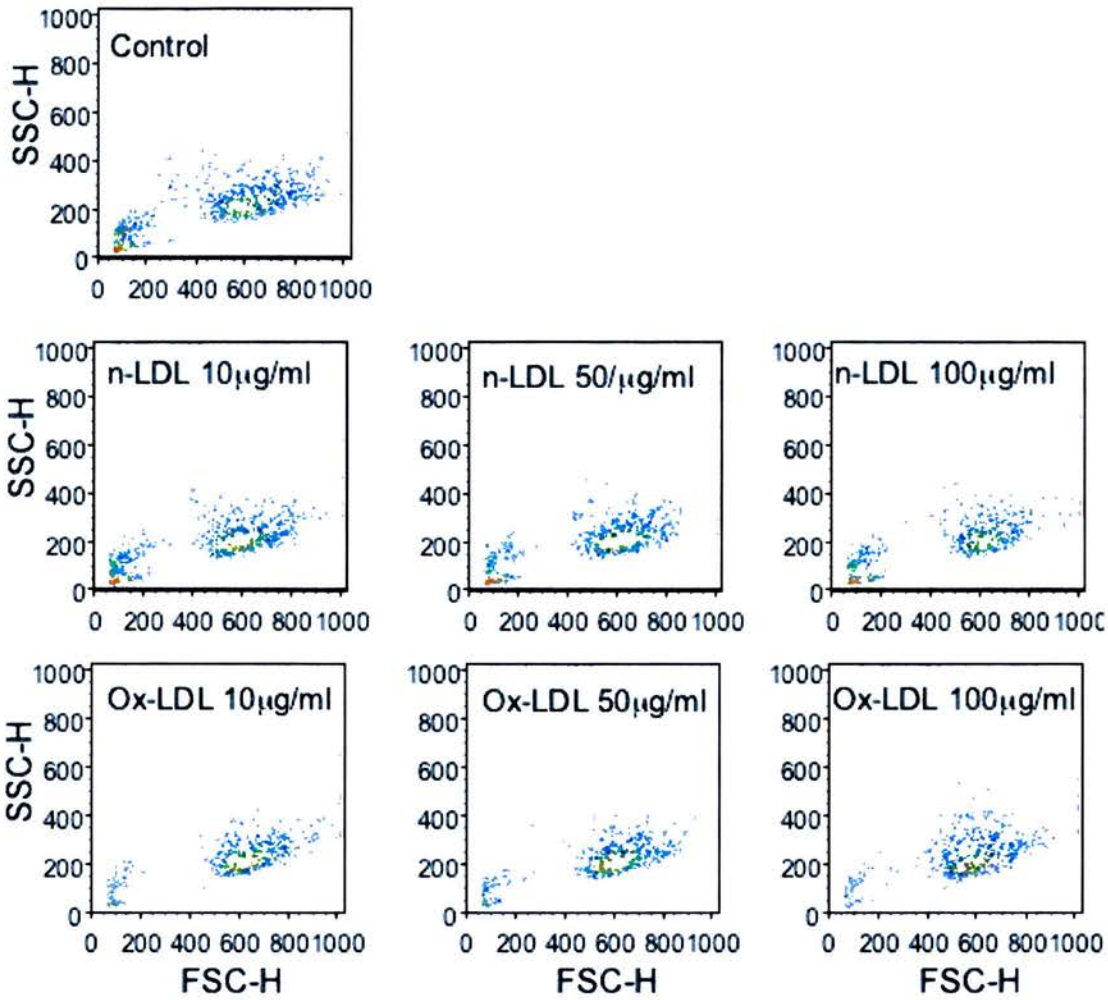


Figure 6-17 Scatter characteristics of LDL supplemented monocytes, prolonged suspension culture

Monocytes cultured in suspension for 5 days in serum-replete control media or media supplemented with increasing concentrations of native or oxidised LDL.

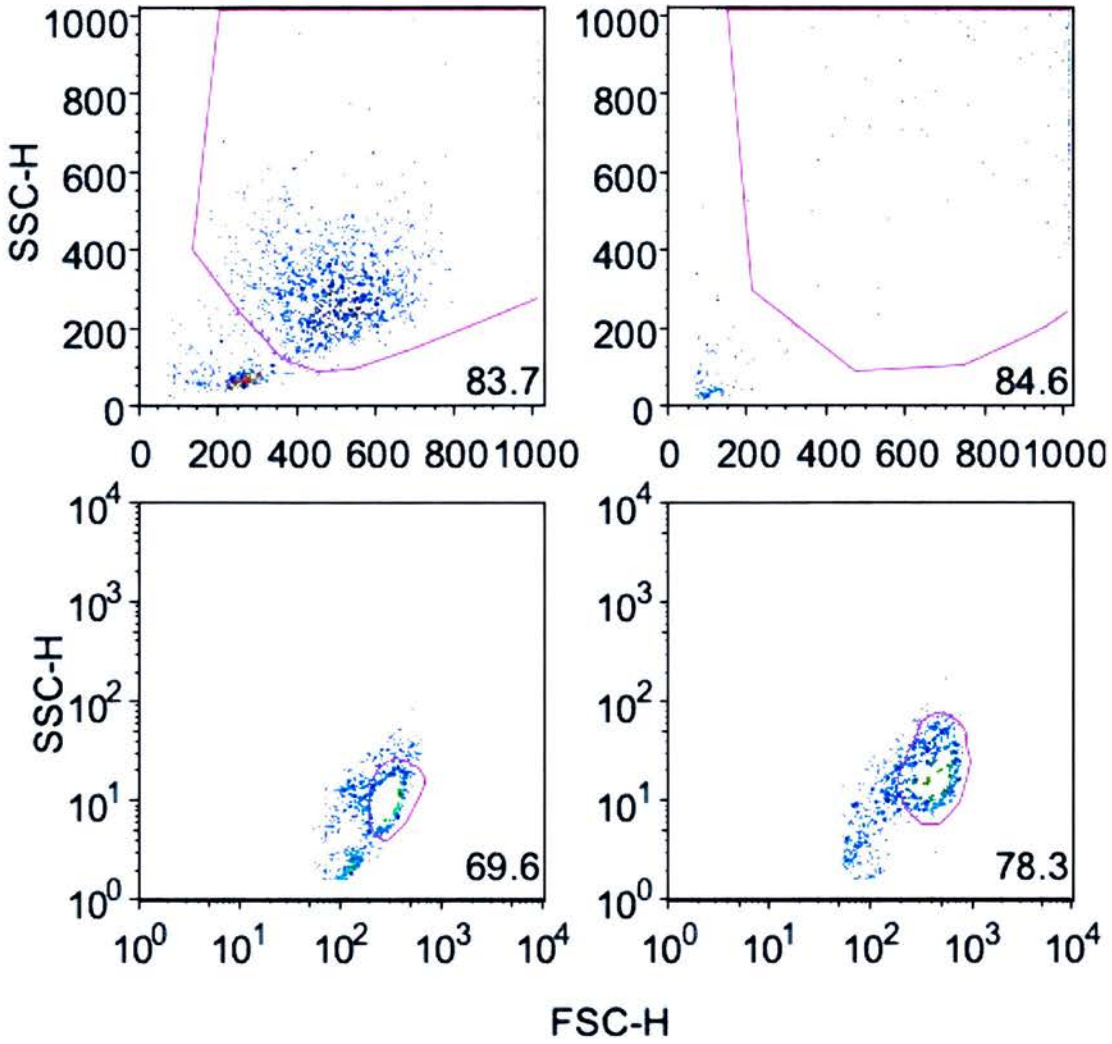


Figure 6-18 Monocyte scatter properties, adherent culture, LDL supplements

Monocyte scatter properties were assessed using linear scale scatter for early adherent culture at 48hrs (upper left panel). Prolonged adherent culture for 8 days altered scatter characteristics, reducing visible cell populations on a linear scale (upper right panel).

Log-scale scatter analysis at 48hrs (lower left panel) and 8 days (lower right panel) allows visualisation of all cell populations, whilst still enabling the exclusion of low forward scatter non-viable cells.

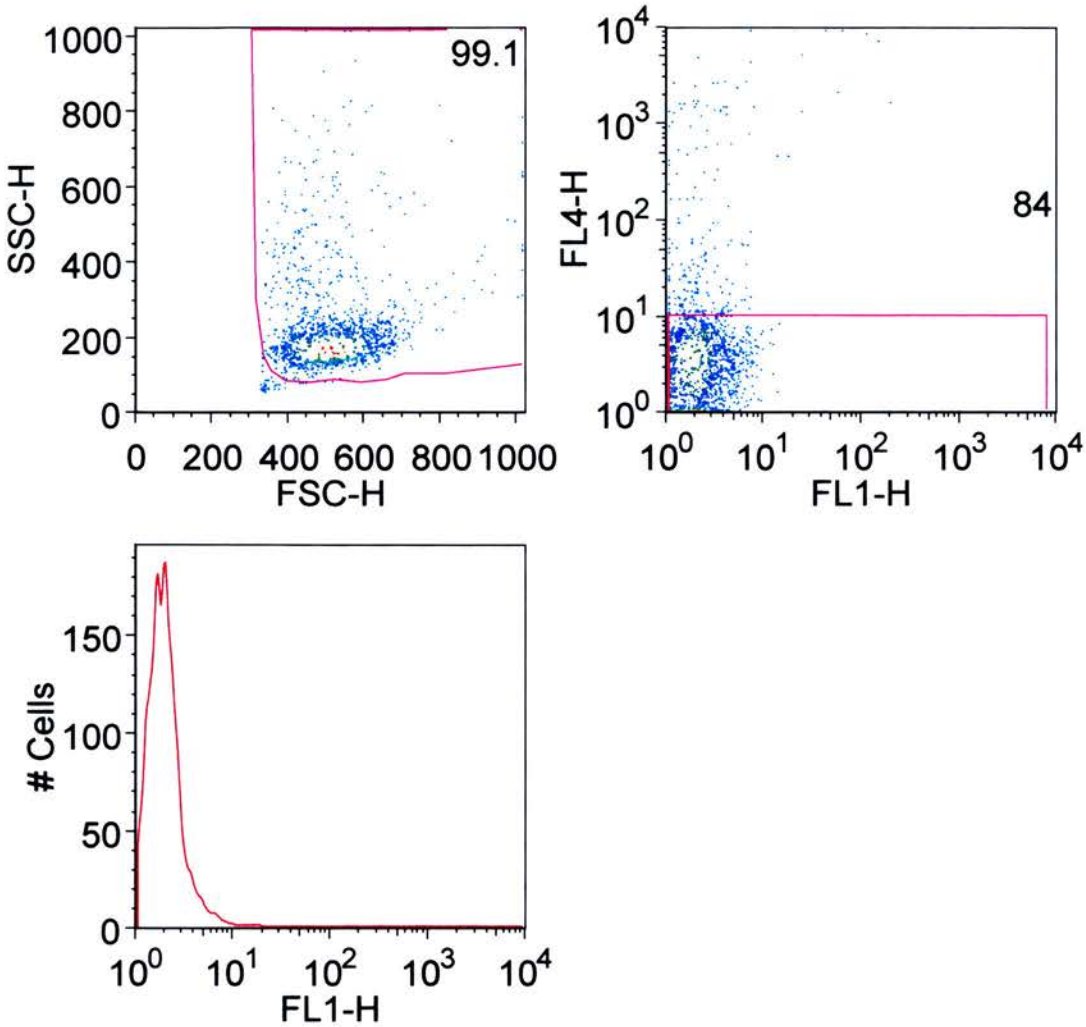


Figure 6-19 Derivation of flow data, suspension culture monocytes

Monocytes cultured in suspension were gated by side scatter (upper left panel) and the uptake of ToPro3 assessed using the FL-4 channel (upper right panel). Cells that excluded ToPro3 were used for flow quantification (lower panel, control sample shown).

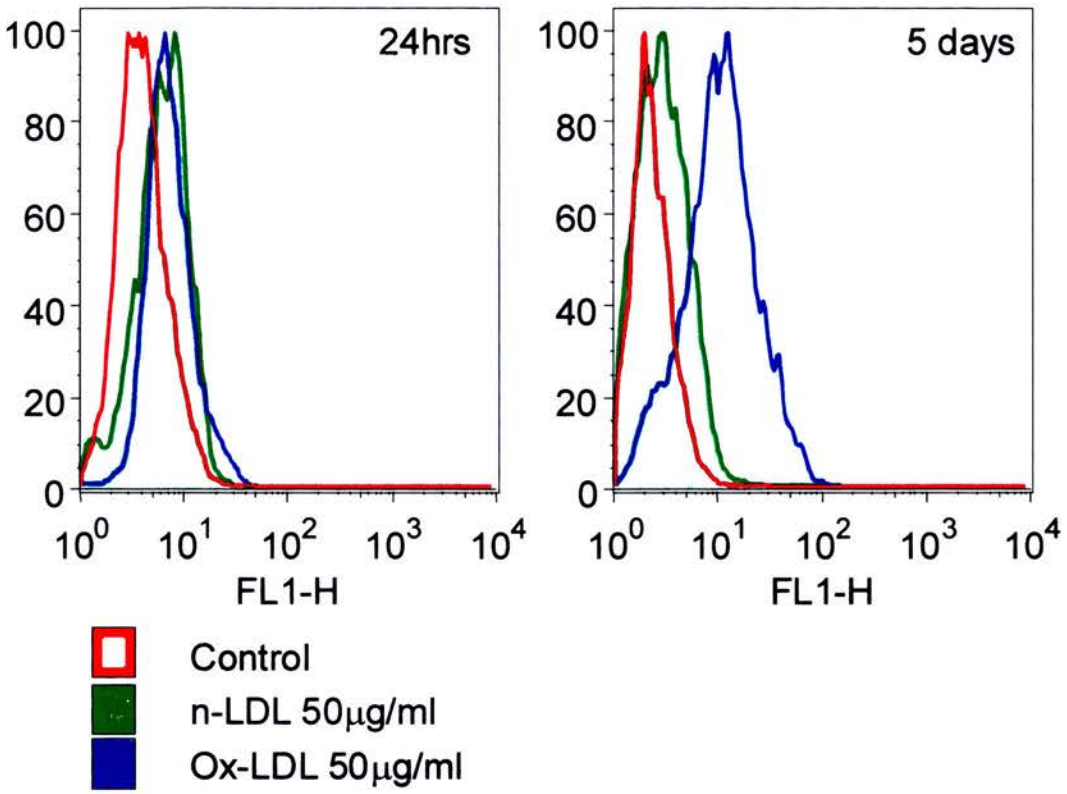


Figure 6-20 Monocyte autofluorescence induced by modified LDL

Both n-LDL and ox-LDL exposure enhance monocyte basal fluorescence levels (left panel). Prolonged culture with ox-LDL differentially increases autofluorescence in contrast to n-LDL (right panel).

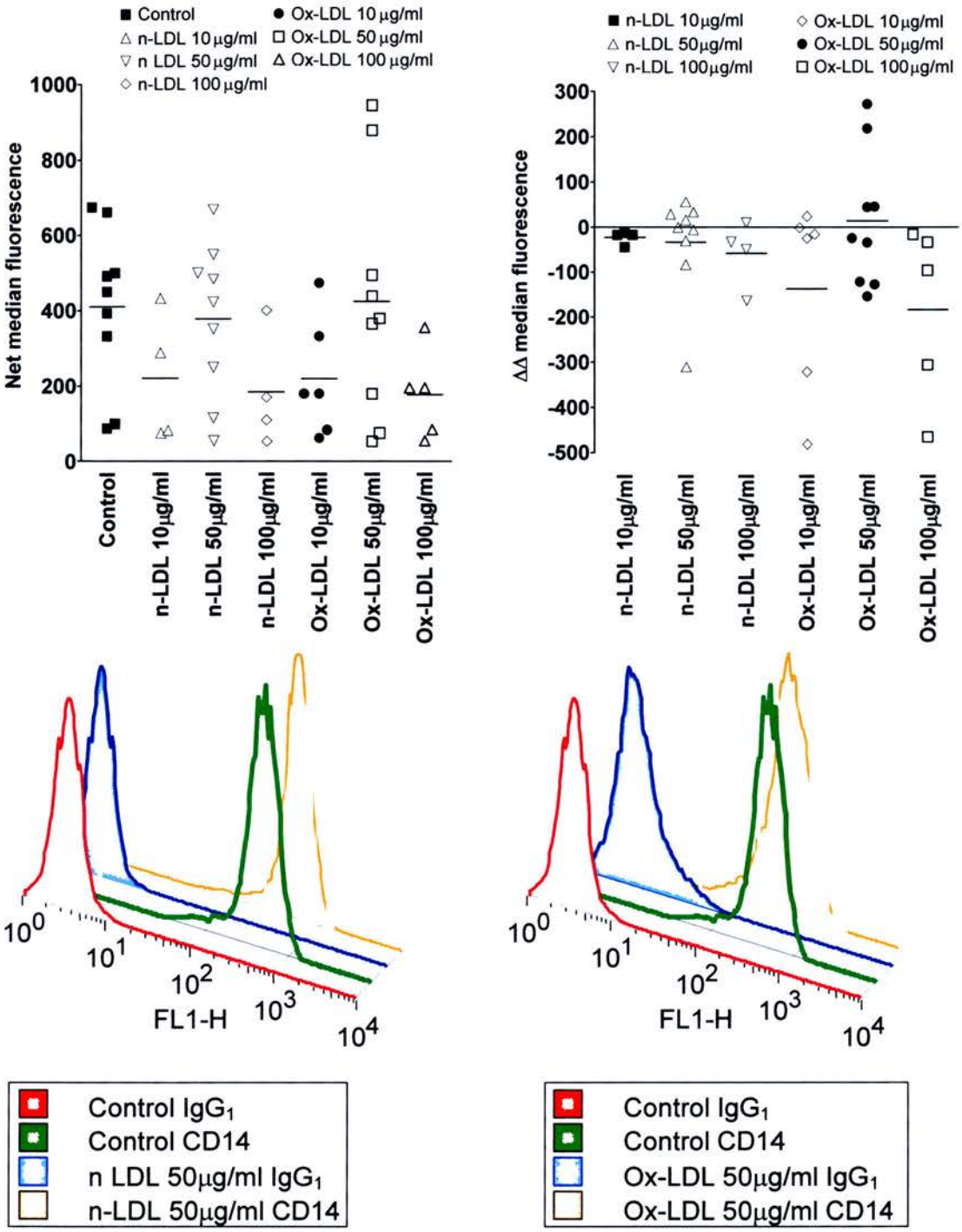


Figure 6-21 Monocyte CD14 expression after 24hrs in suspension culture with lipid supplementation

No changes in net median CD14 fluorescence (upper left panel, one-way ANOVA, n=4 to 9, p=0.1517) or net fluorescence relative to control (upper left panel, one-way ANOVA, n=4 to 9, p=0.3555) were noted following LDL-supplemented suspension culture for 24 hours. Representative histograms are shown for the 50µg/ml treatment, with no alteration in CD14 fluorescence seen. Histograms are offset to allow visualisation of overlapping signals.

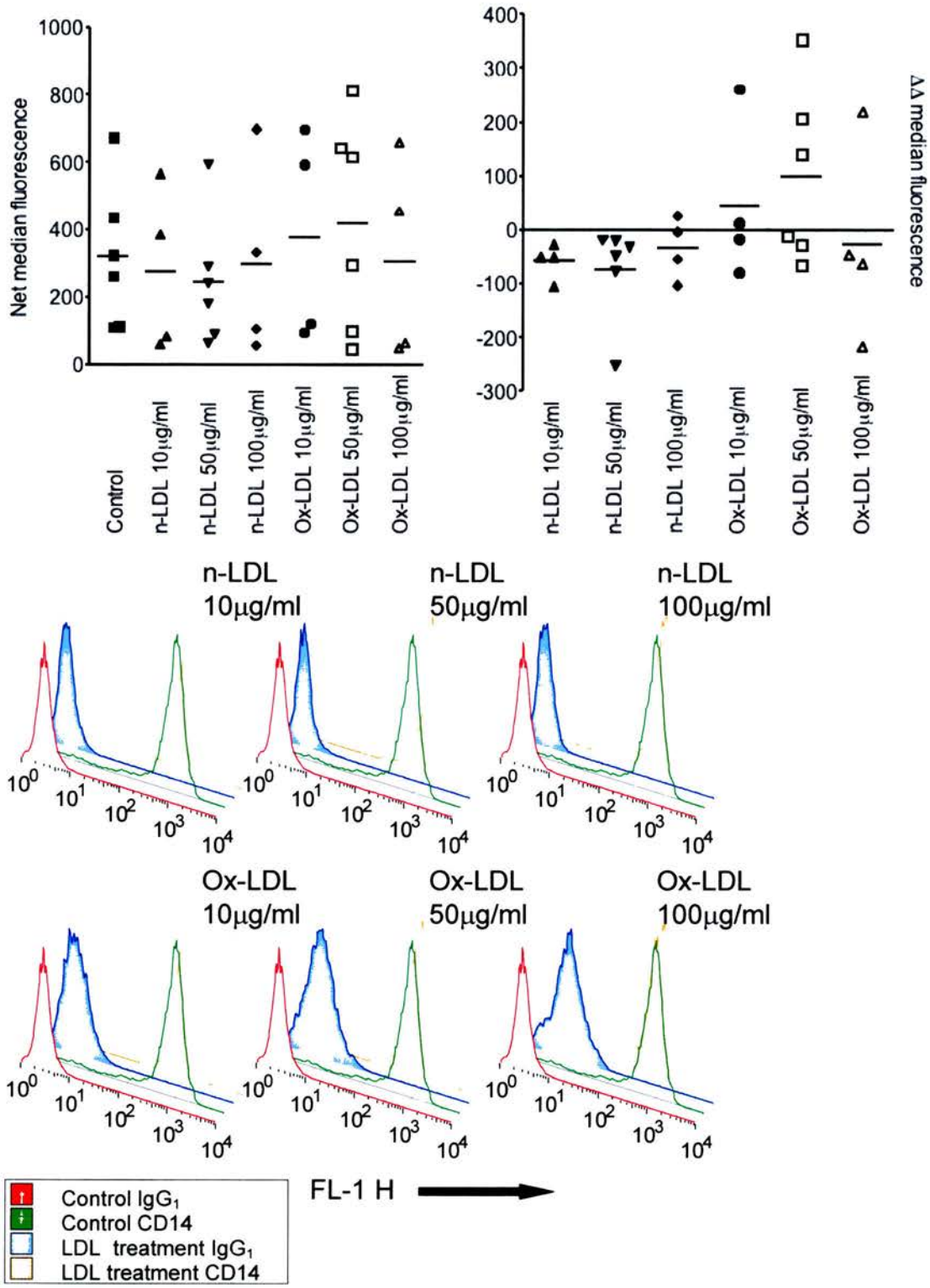


Figure 6-22 Monocyte CD14 expression, suspension culture 72hrs, LDL supplements

CD14 expression is unchanged in monocytes cultured with n-LDL or Ox-LDL at 72 hours (net median fluorescence, one-way ANOVA, n=4 to 5, p=0.9999; fluorescence relative to control, one-way ANOVA, n=4, p=0.9893).

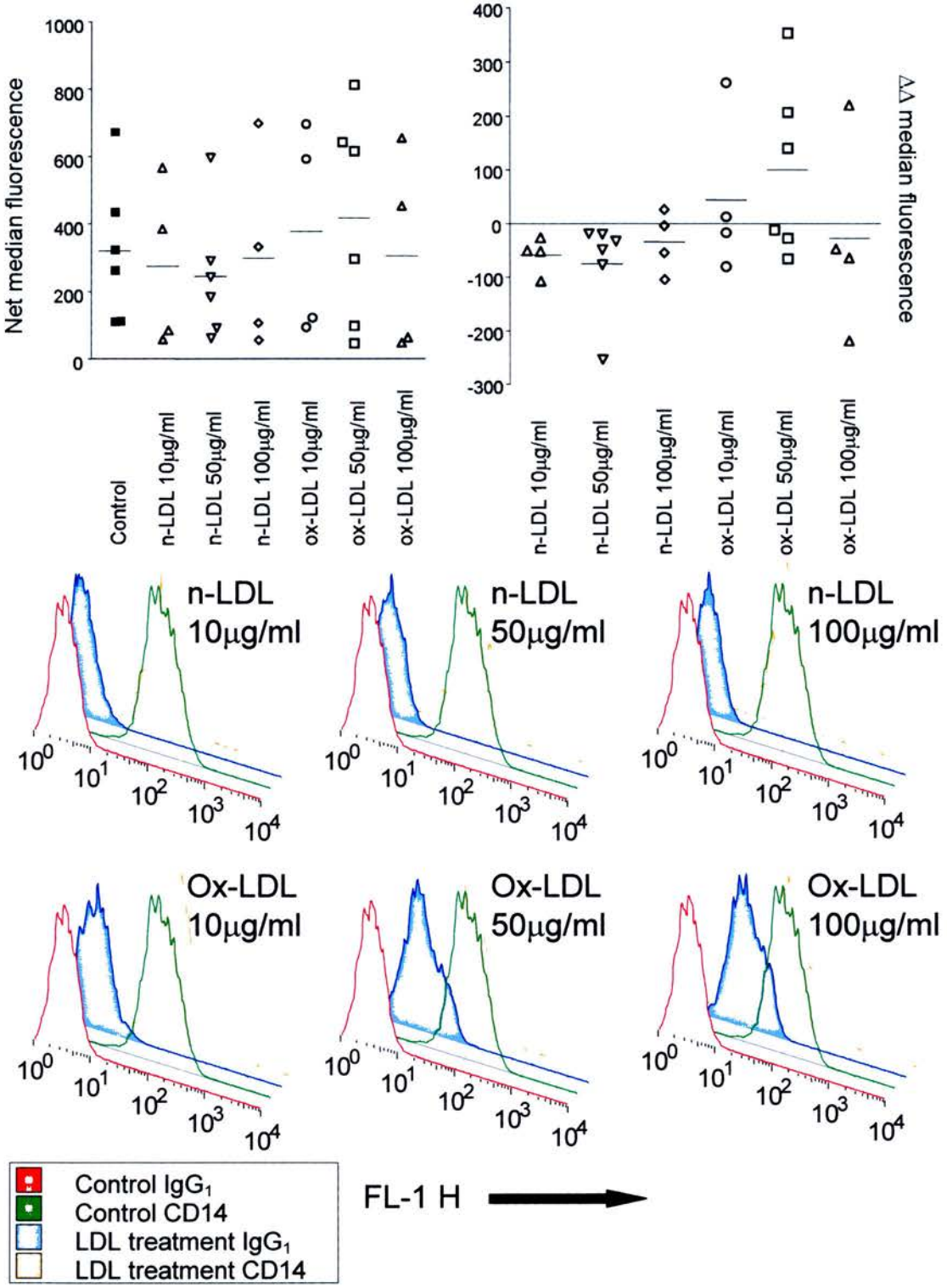


Figure 6-23 Monocyte CD14 expression, suspension culture, 5 days, LDL supplements

No significant changes in net median CD14 fluorescence (upper left panel, one-way ANOVA, n=4 to 6, p=0.2131) or in net fluorescence relative to control (upper right panel, one-way ANOVA, n=4 to 6, p=0.2926) were induced by LDL exposure.

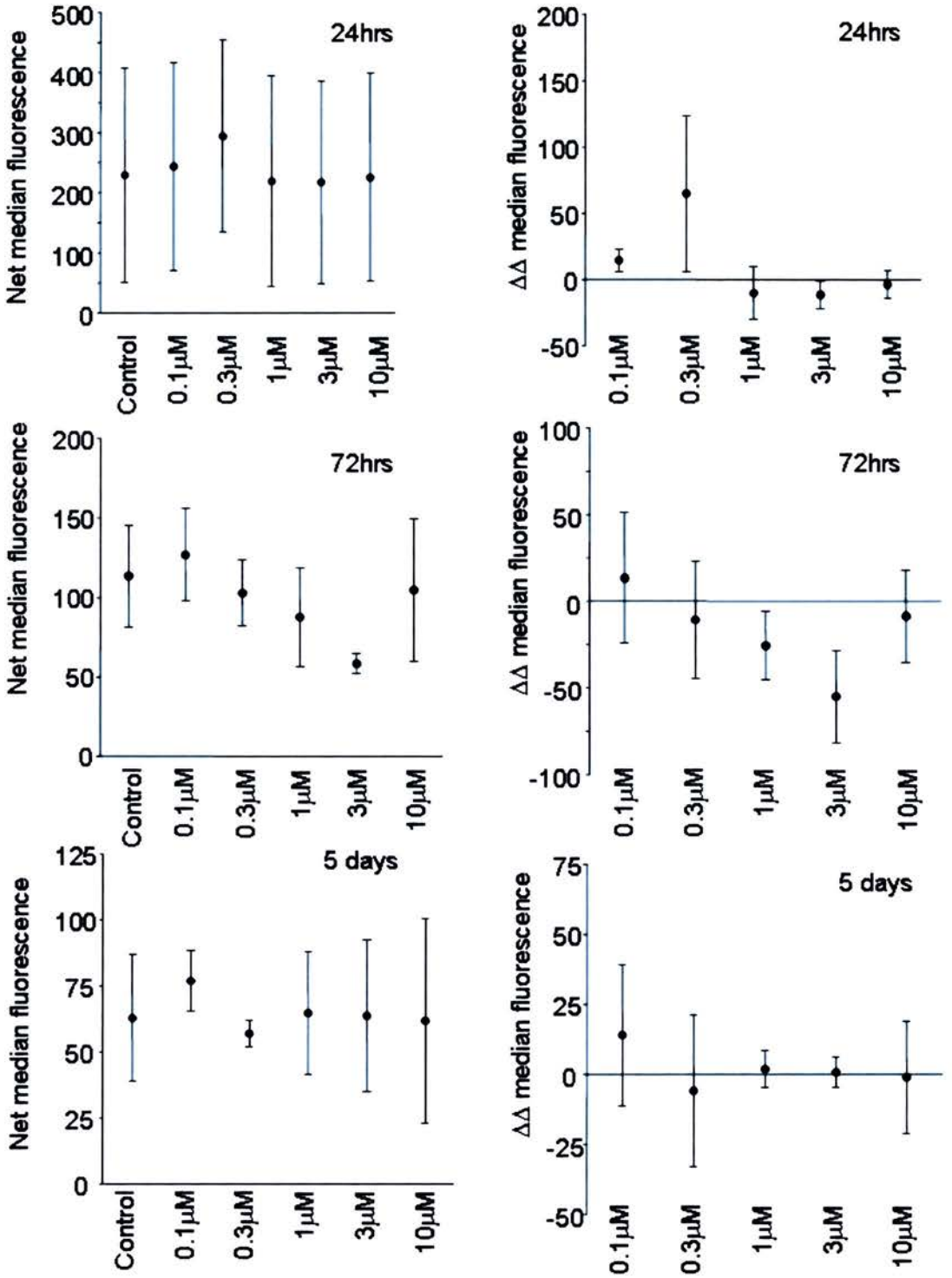


Figure 6-24 Monocyte CD14 expression, suspension culture, 15dPGJ$_2$ supplements

No significant alterations in net median CD14 expression (left hand panels) or CD14 expression relative to control (right hand panels) were noted during prolonged culture with 15dPGJ$_2$ at increasing concentrations over a 3-log concentration range, (n=3, for full data analysis see supplementary data, Tables 6.5-6.7).

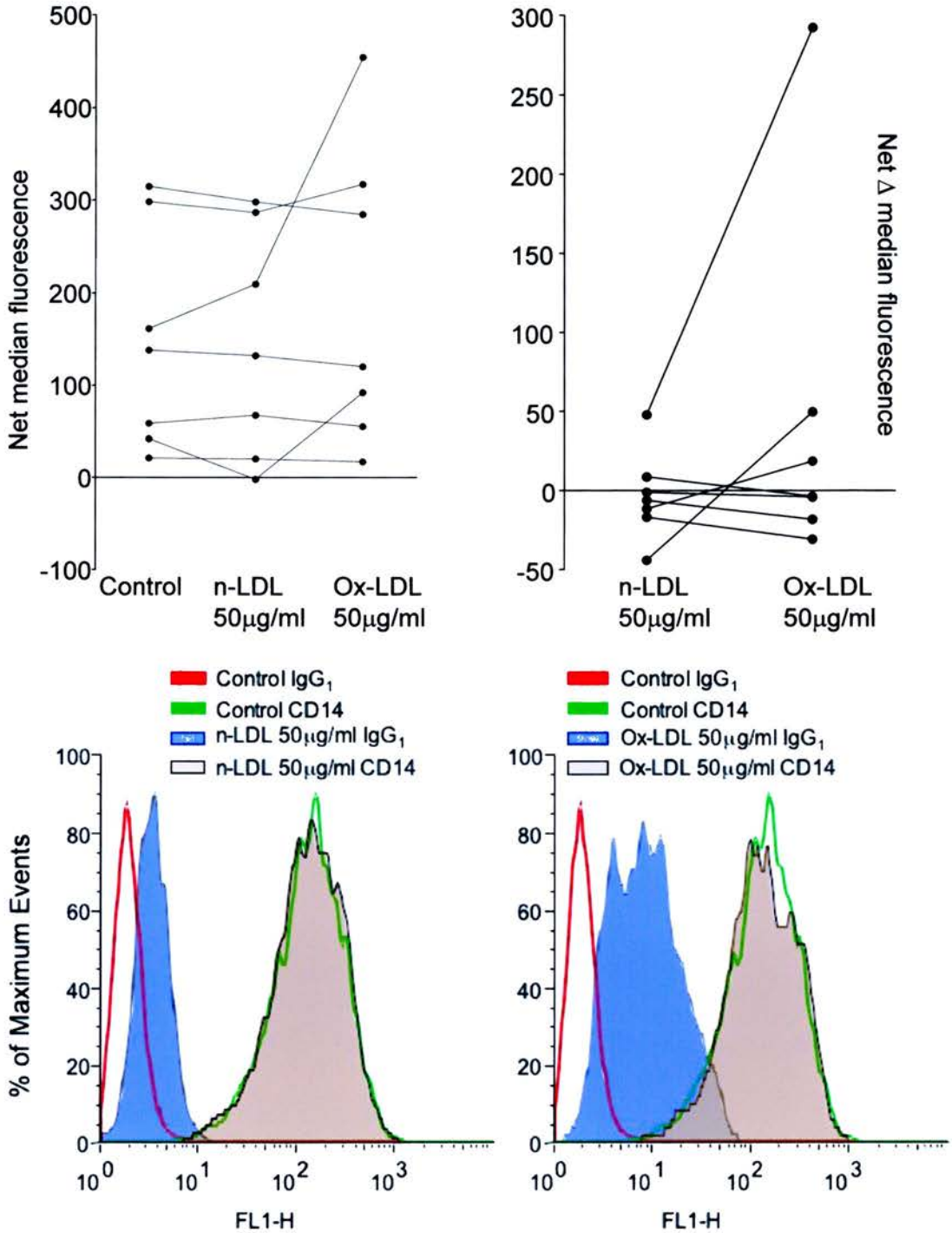


Figure 6-25 Monocyte CD14, adherent culture, 48hrs, LDL supplements

Early of adherent monocytes phenotyping in 50μg/ml n-LDL or ox-LDL showed no significant alteration in net median CD14 fluorescence (upper left panel, one-way ANOVA, n=7, p=0.7682) or net fluorescence relative to control (upper right panel, Wilcoxon signed rank test, n=7, p=0.3438). Representative histogram overlays for n-LDL and ox-LDL are shown in the lower panels.

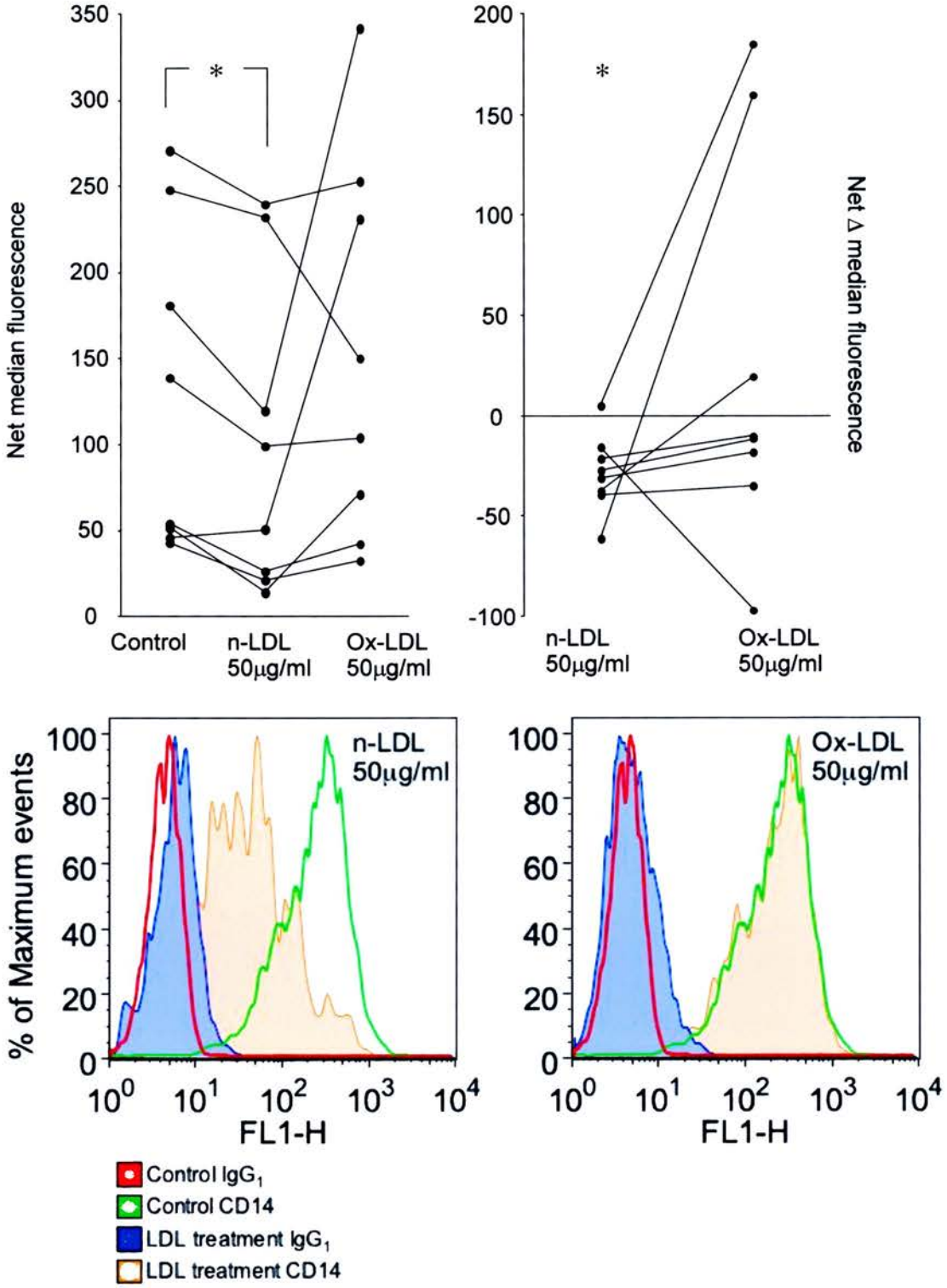


Figure 6-26 Monocyte CD14 expression adherent culture 8 days, LDL supplements

n-LDL reduced total CD14 expression (n=8, p=0.0303, upper left panel), and CD14 expression relative to control (n=8, p=0.0078, upper right panel) after prolonged culture. Ox-LDL produced no change in relative fluorescence (n=8, p=0.4727). Histogram overlays suggested stable CD14 expression (lower panels).

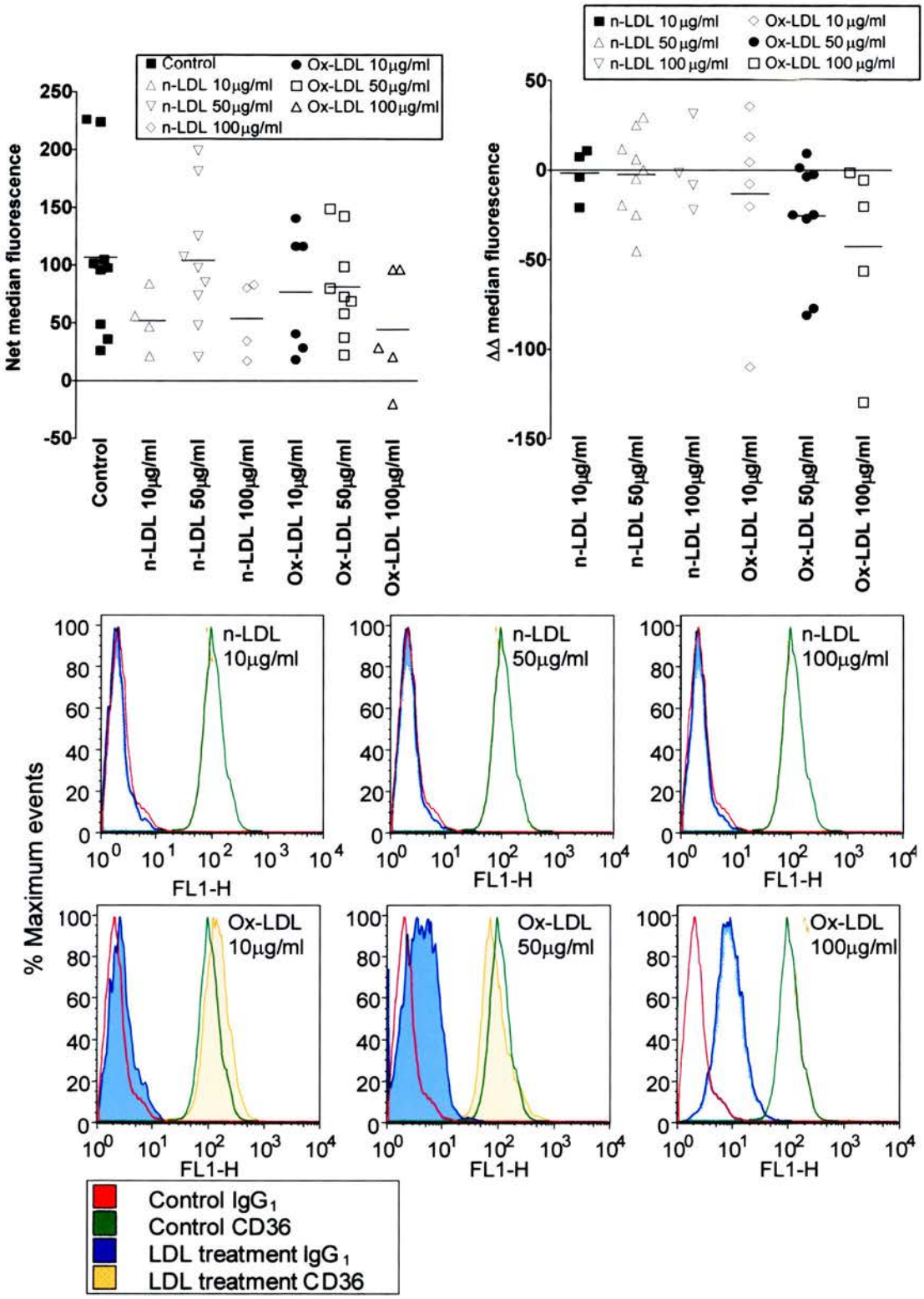


Figure 6-27 Monocyte CD36 expression, 24hrs, suspension culture, LDL supplements

No changes in net median CD36 expression were noted (one-way ANOVA, n=4 to 9, p=0.2656) or net fluorescence relative to control (n=4 to 9, p=0.3813) following LDL exposure.

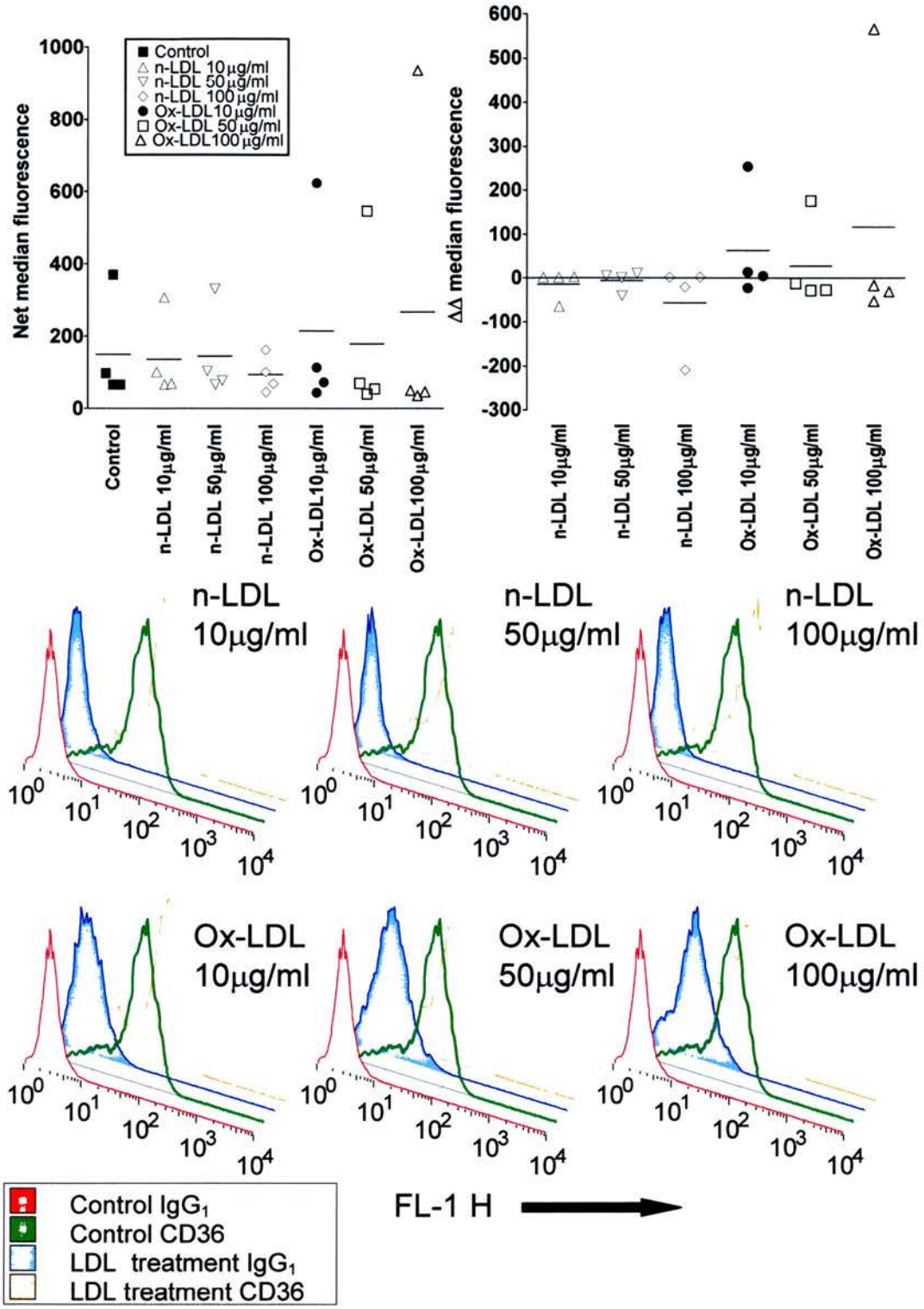


Figure 6-28 Monocyte CD36 expression, 72hrs suspension culture, LDL supplements

No changes in monocyte net median CD36 fluorescence (one-way ANOVA, n=4, p=0.3343) or net fluorescence relative to control (n=4, p=0.2926) were noted. Overlay plots are offset to display histograms.

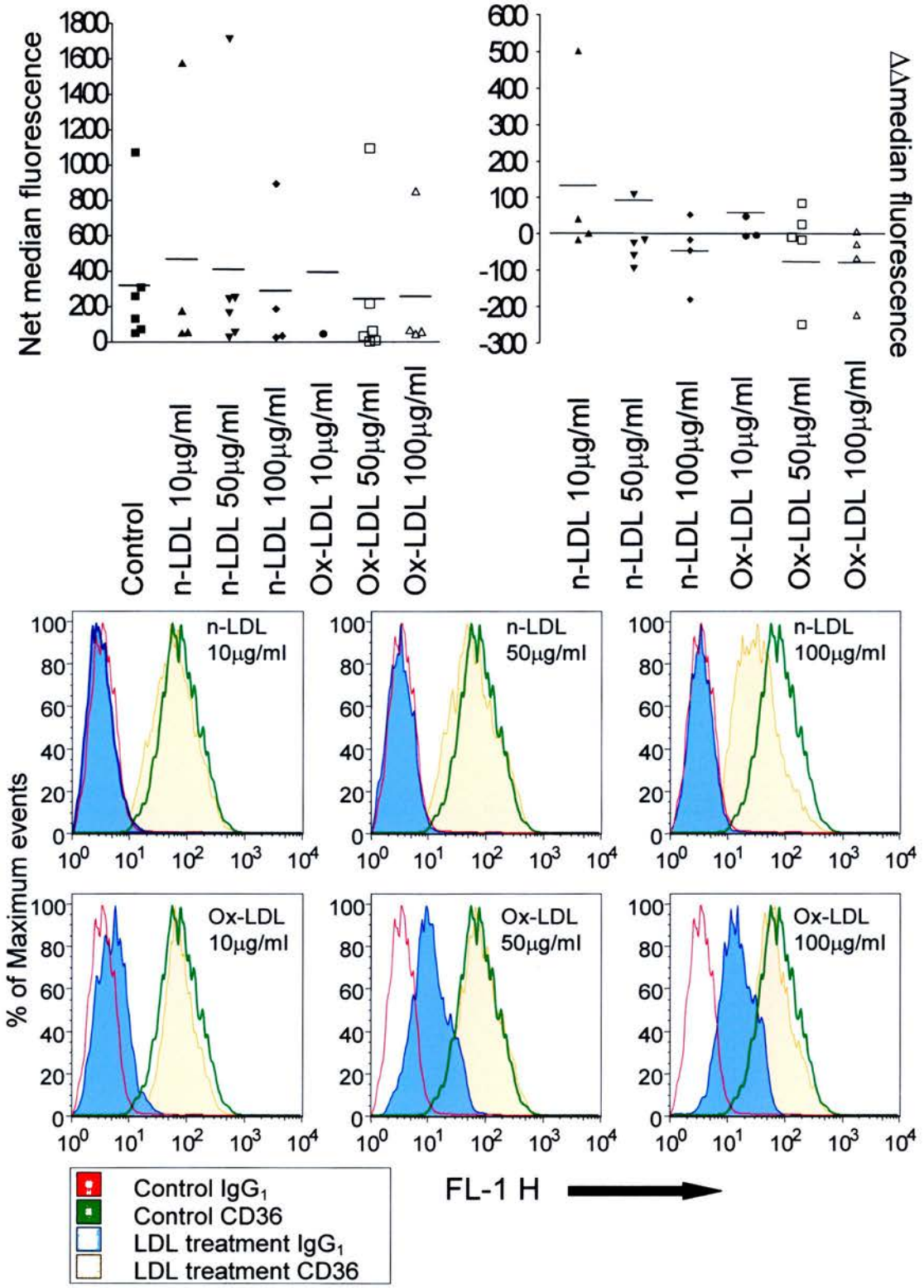


Figure 6-29 Monocyte CD36 expression, 5 days, suspension culture, LDL supplements

No changes in net median fluorescence (upper left panel, n=4 to 6, p=0.6815) or net median fluorescence relative to control (right left panel, n=4 to 6, p=0.6126) were apparent with LDL exposure in suspension culture at 5 days.

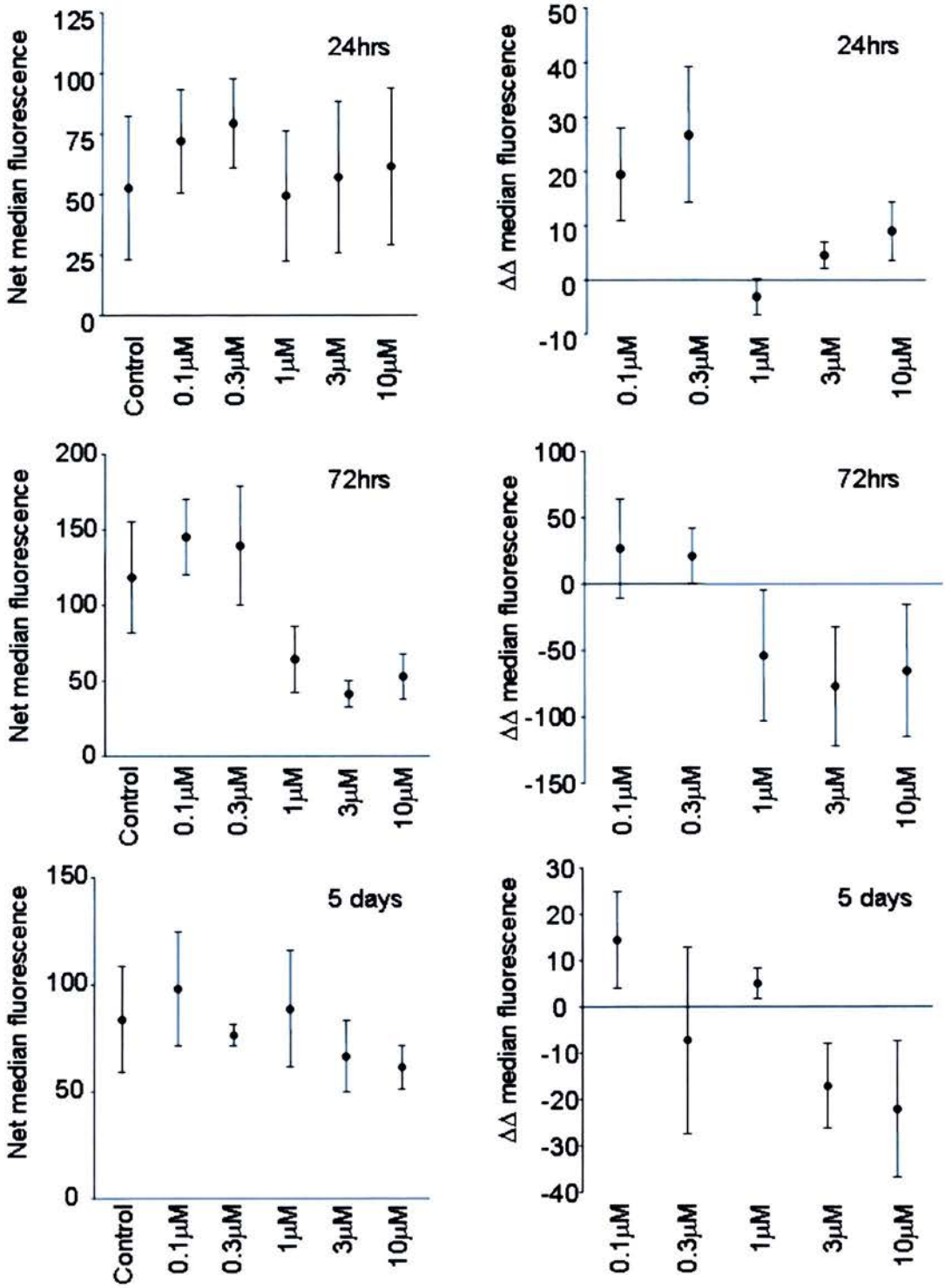


Figure 6-30 Monocyte CD36 expression, suspension culture, 15dPGJ$_2$ supplements

No alterations in net median CD36 fluorescence or fluorescence relative to control were apparent following 15dPGJ$_2$ exposure, at 24hrs, 72hrs and 5 days (n=3, all non-significant, see supplementary data, Tables 6.13-6.15 for full analyses).

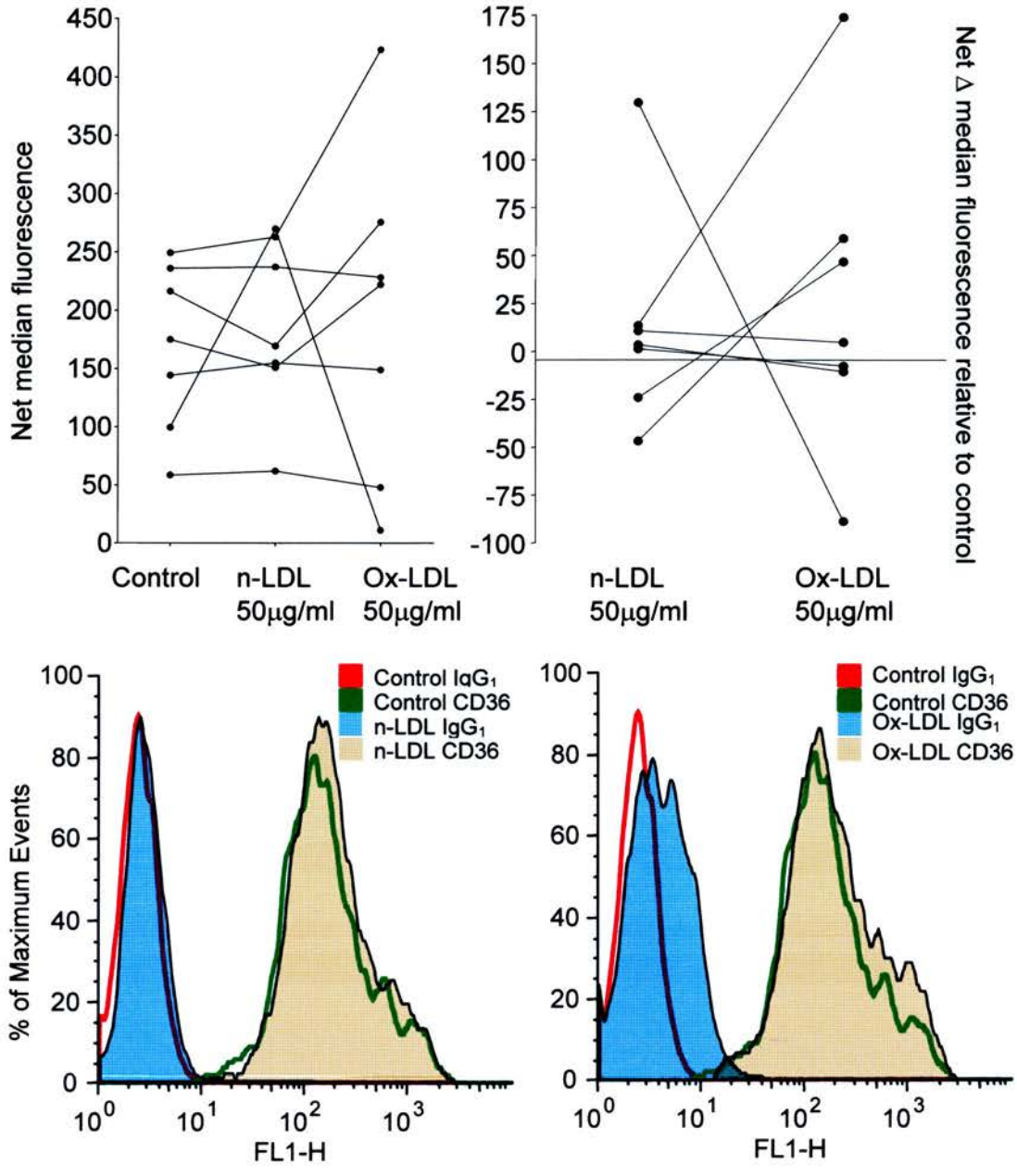


Figure 6-31 Monocyte CD36 expression, adherent culture, 48 hours, LDL supplements

Adherent monocytes were treated with native or oxidised LDL at 50µg/ml for 48 hours. No significant change was noted in net median CD36 fluorescence (upper left panel, one-way ANOVA, n=7, p=0.6197), or fluorescence relative to control (upper right panel, Wilcoxon signed rank test, n=7, p=0.3438). Representative histograms display stable CD36 expression for both native LDL (lower left panel) and Ox-LDL (lower right panel) at this time point.

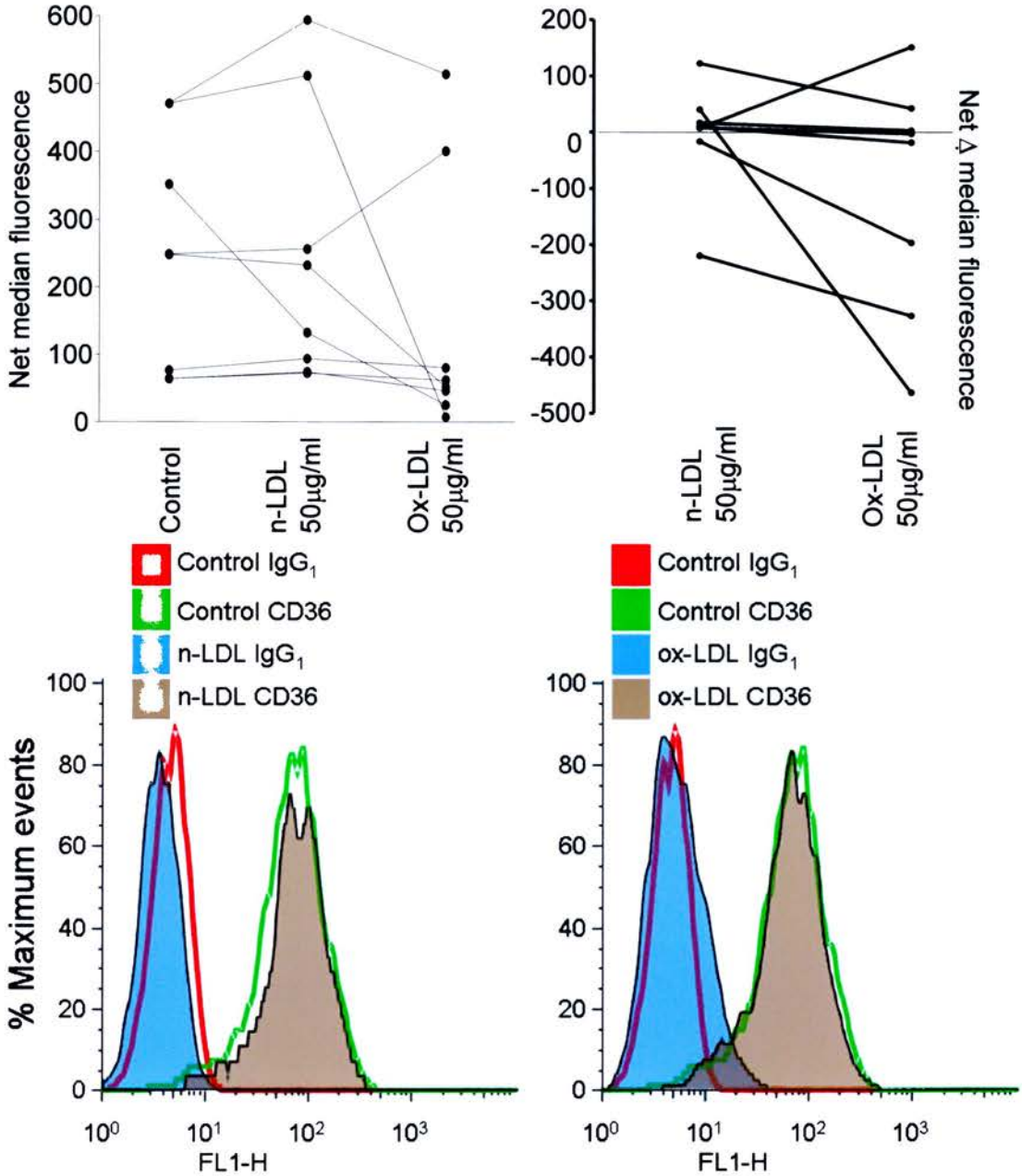


Figure 6-32 Monocyte CD36 expression, day 8, adherent culture, LDL supplements

No statistically significant difference was seen in net median CD36 expression (upper left panel, one-way ANOVA, n=8, p=0.0789), or relative fluorescence to control (upper right panel, Wilcoxon signed rank test n=8, p=0.1914) after prolonged adherent culture in n-LDL or ox-LDL at 50µg/ml.

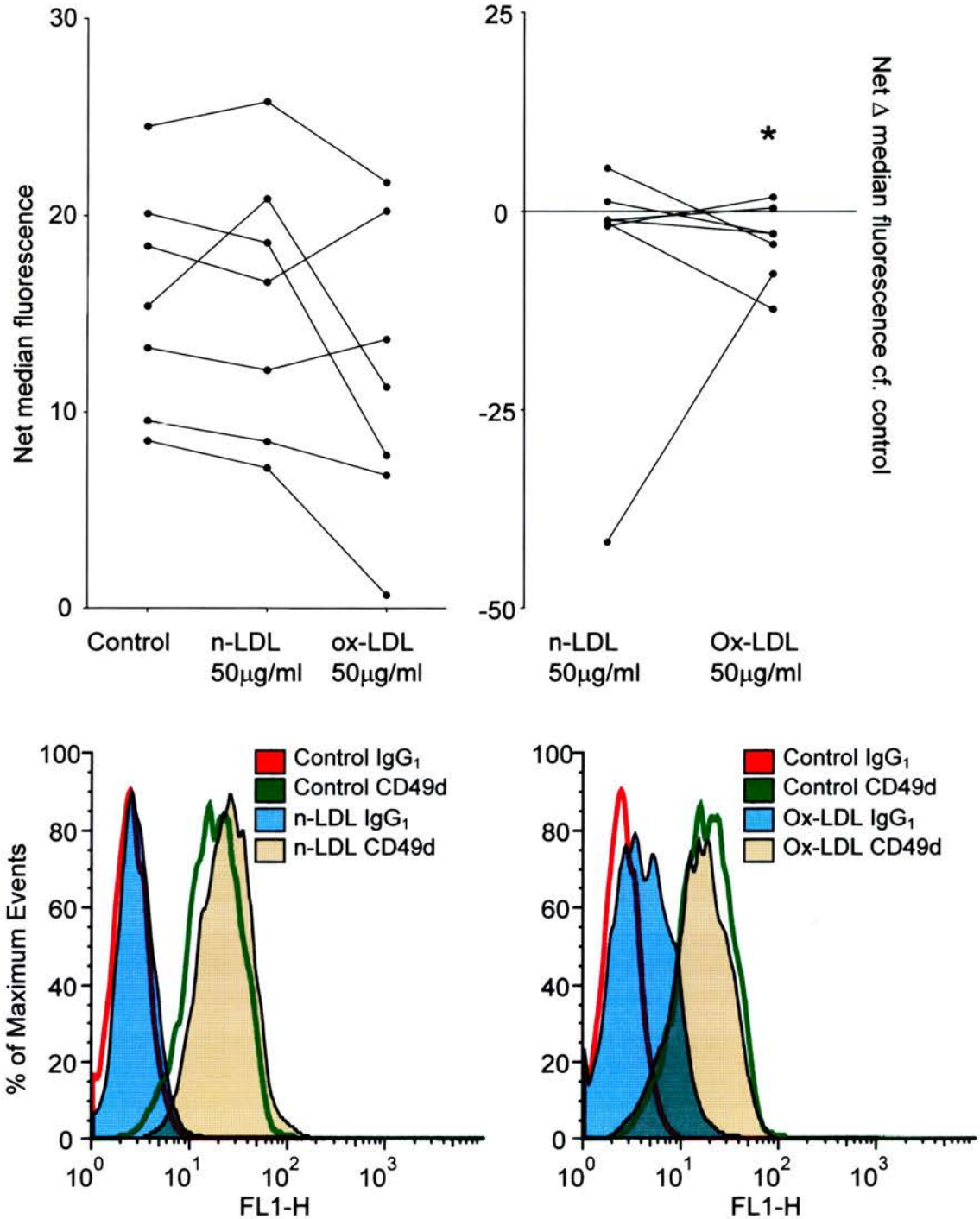


Figure 6-33 Monocyte CD49d expression, adherent culture, 48hrs, LDL supplements

No significant change in total net median expression of CD49d occurred after 48 hours of adherent culture with either n-LDL or ox-LDL supplementation (one-way ANOVA, n=7, p=0.3046, upper left panel). A decrement in net median fluorescence relative to control was suggested by statistical analysis (Wilcoxon signed rank test, n=7, p=0.0391, upper right panel), but was not obvious in histogram overlays, with signals at the lower end of the dynamic range.

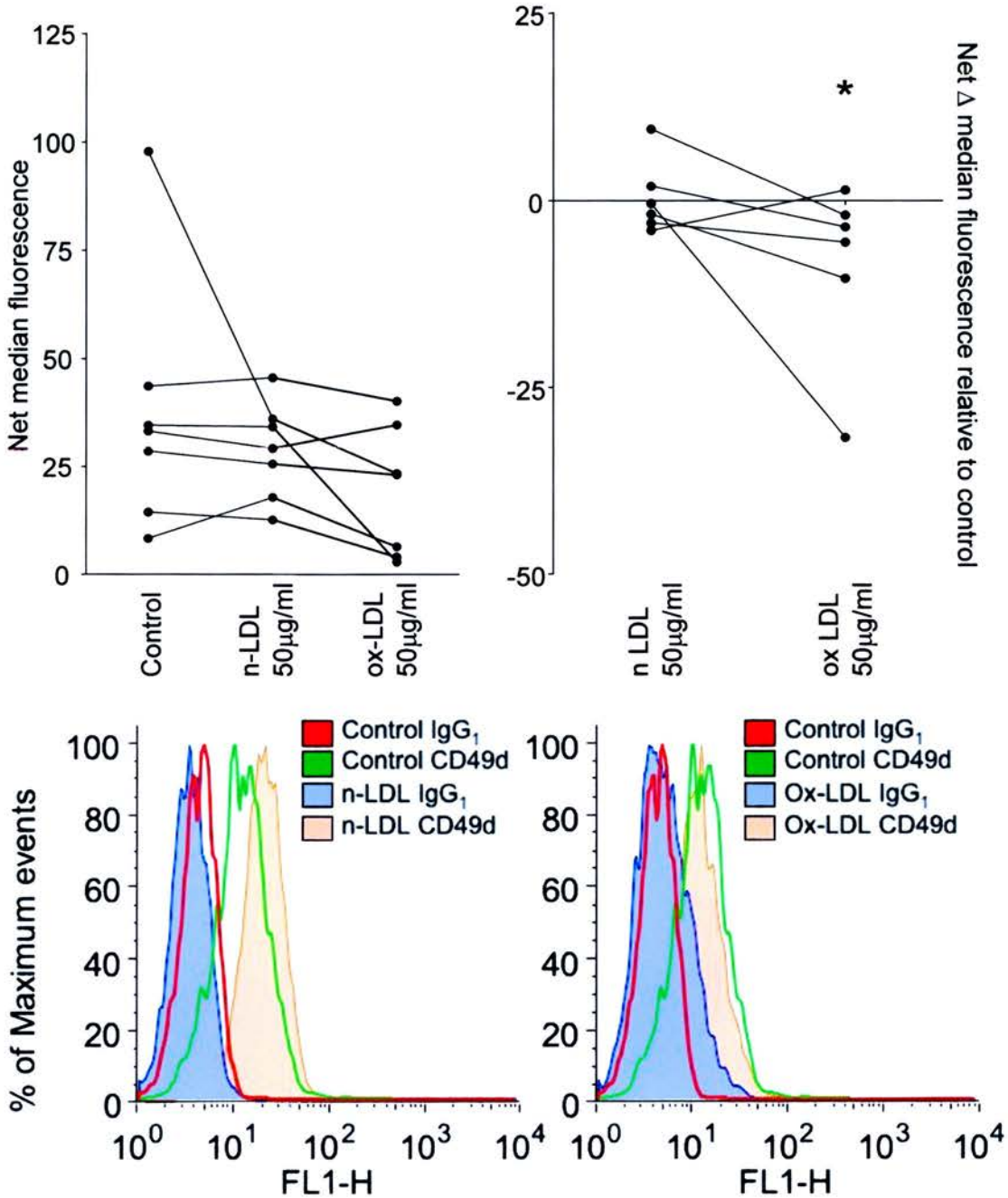


Figure 6-34 Monocyte CD49d expression, day 8, adherent culture, LDL supplements

Prolonged adherent culture with Ox-LDL at 50µg/ml did not significantly reduce net median monocyte CD49d expression, (upper left panel, one-way ANOVA n=7, p=0.0515), although no alteration was noted with respect to n-LDL. Net changes in fluorescence relative to control were significant for ox-LDL alone (upper right panel Wilcoxon signed rank test, n=7, p=0.0156). The histogram overlays suggest that actual alterations in fluorescence were small, and at the low end of the fluorescence range.

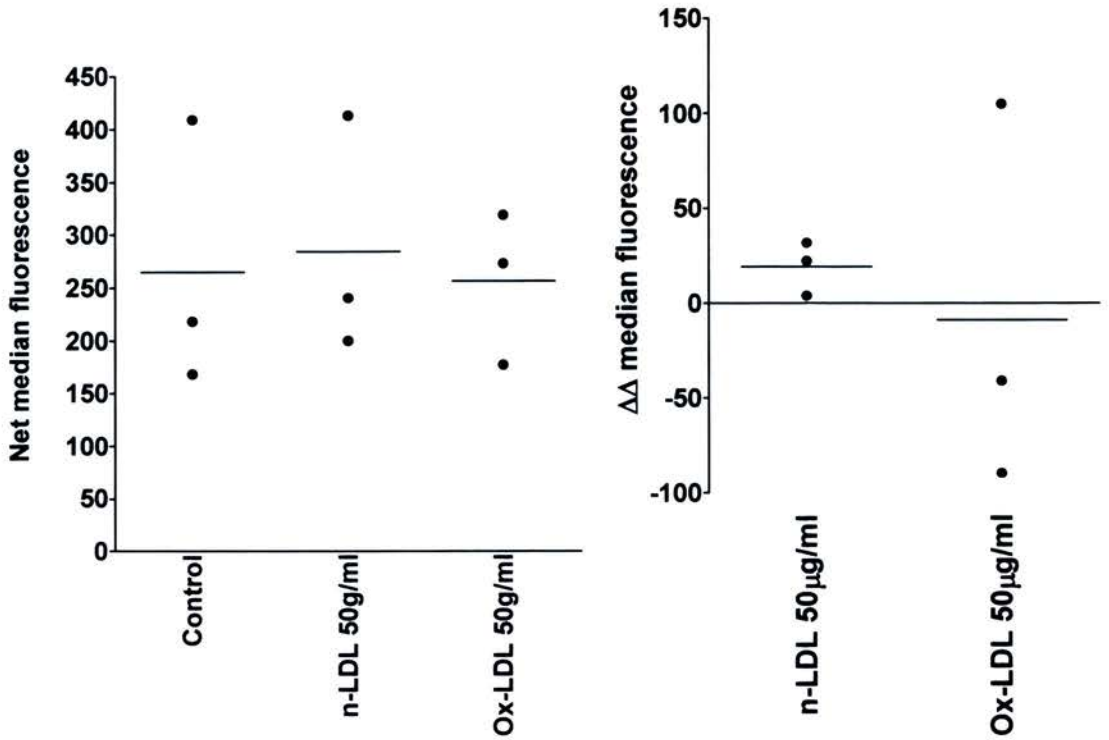


Figure 6-35 Monocyte CD11b, 24hrs suspension culture, LDL supplements

No statistically significant alteration in monocyte CD11b expression was seen following LDL exposure (net median fluorescence, n=3, p=0.5278, net median fluorescence relative to control, n=3, p=0.625).

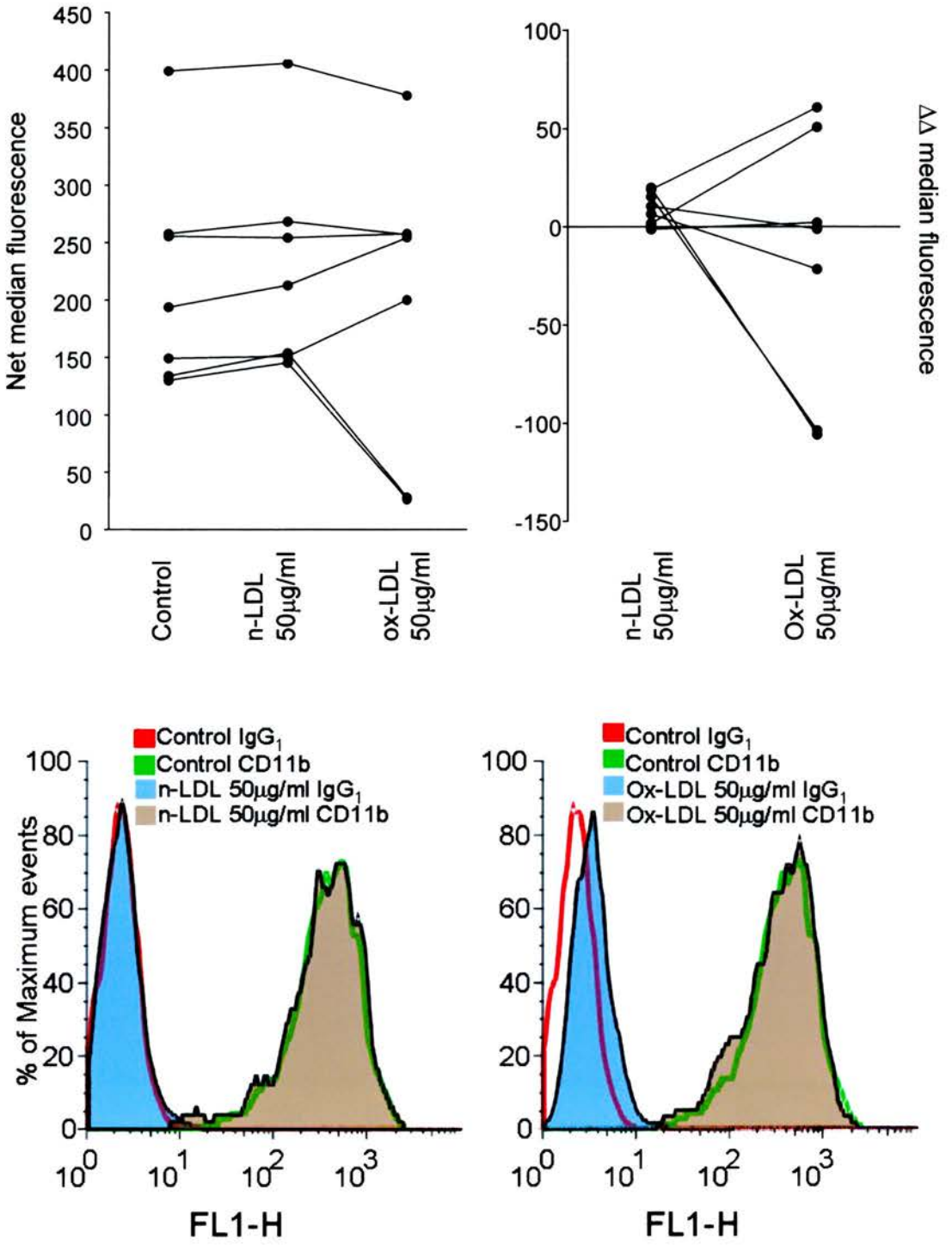


Figure 6-36 Monocyte CD11b expression, adherent culture, 48hrs, LDL supplements

No significant changes were noted in net median CD11b expression (n=7, p=0.4861) following LDL exposure. Net median CD11b fluorescence relative to control appeared increased by CD11b (n=7, p=0.0156) although this was a small change. Ox-LDL did not alter relative CD11b fluorescence compared to control (n=7, p=0.3438).

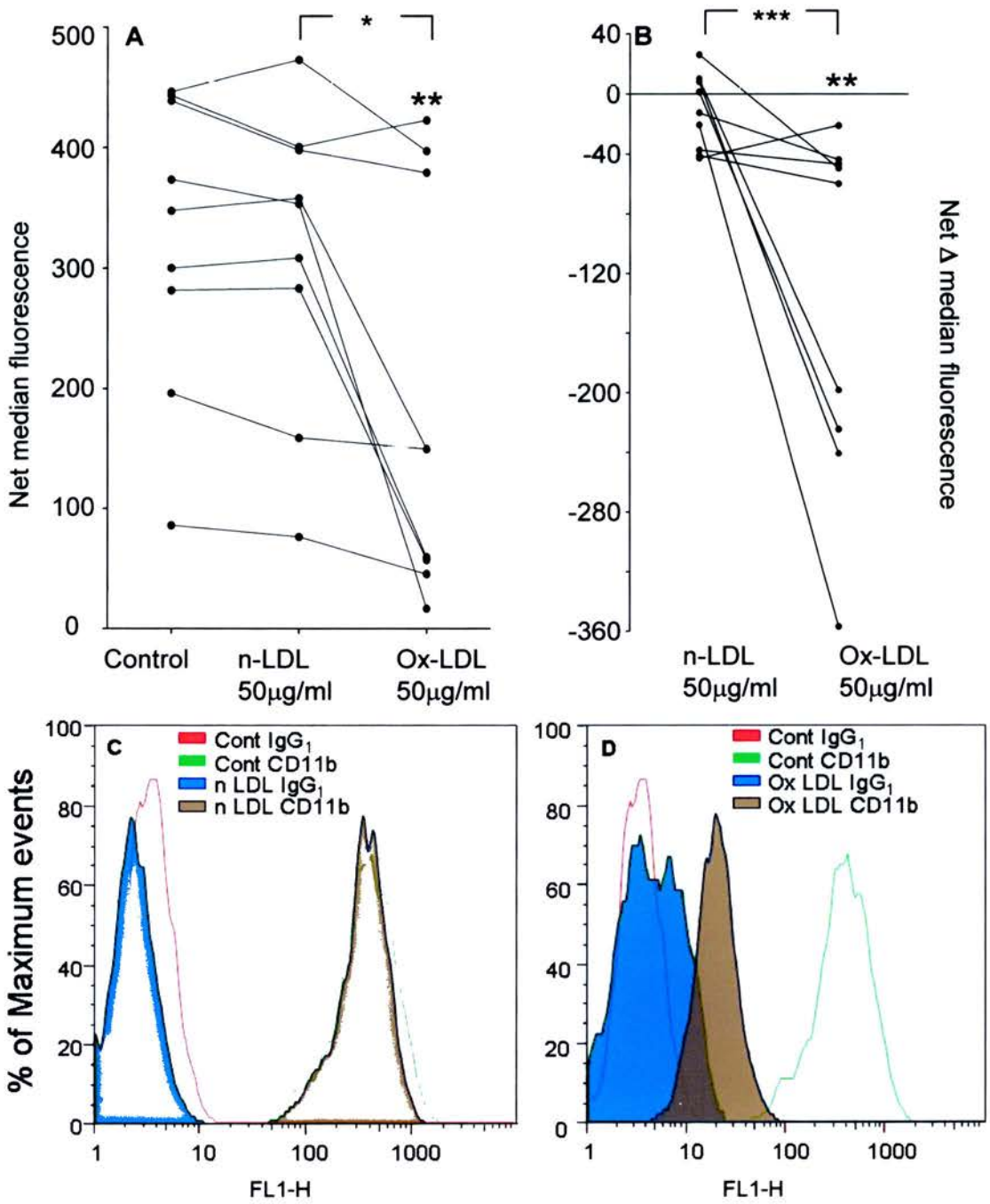


Figure 6-37 Monocyte CD11b expression adherent culture, 8 days, LDL supplementation

Significant reductions of CD11b net total fluorescence (n=9, p=0.0029, panel A), and net fluorescence relative to control (n=9, p=0.002, panel B) occurred following monocyte exposure to ox-LDL but not to n-LDL. Representative histograms illustrate monocyte CD11b expression after n-LDL exposure (panel C) contrasting with reduced CD11b expression following ox-LDL exposure (panel D).

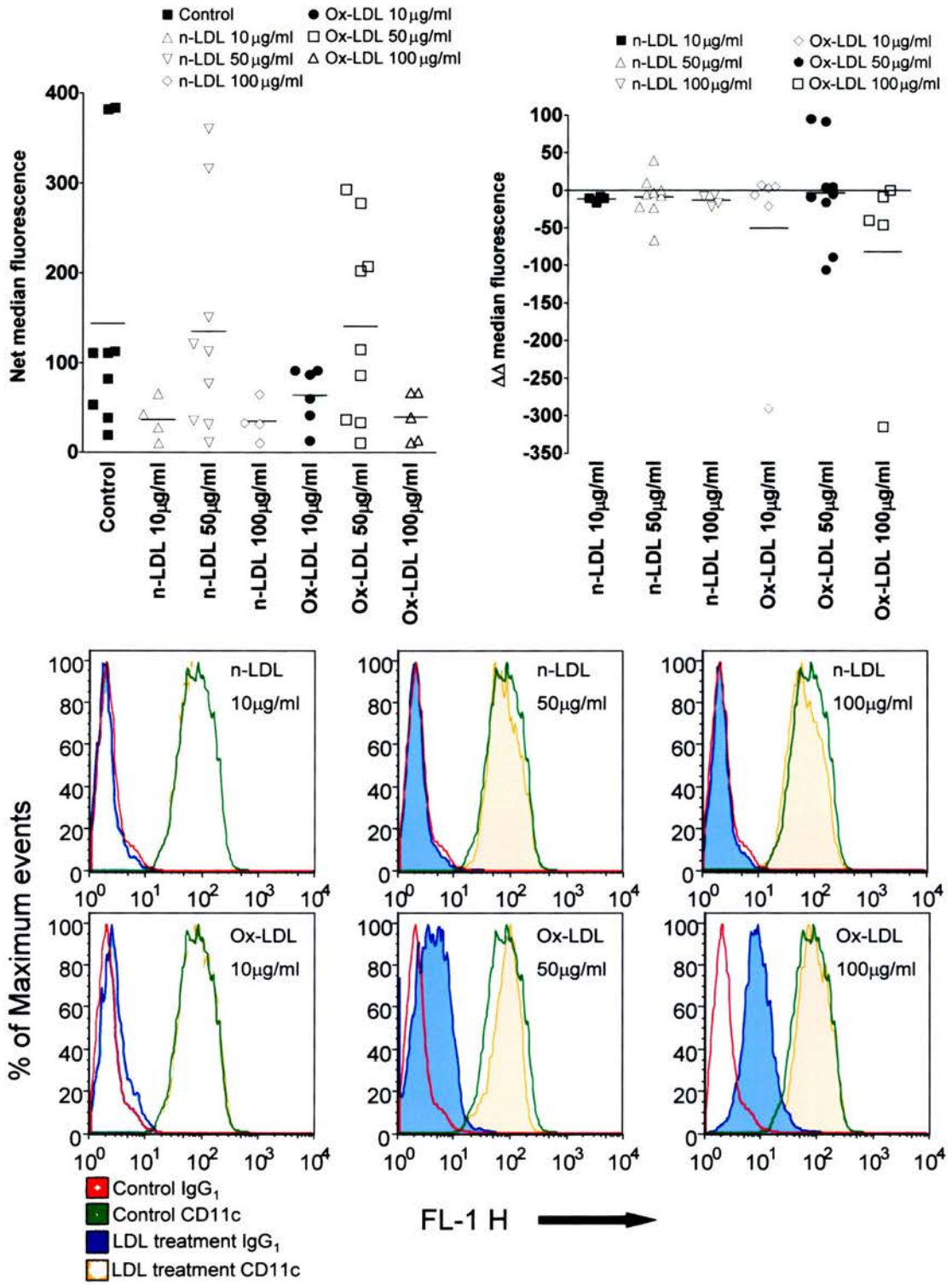


Figure 6-38 Monocyte CD11c expression, 24hrs suspension culture, LDL supplements

No significant alterations in CD11c expression were noted during prolonged suspension culture, for net median fluorescence (one-way ANOVA, n=4 to 9, p=0.0906) or for net fluorescence relative to control (one-way ANOVA, n=4 to 9, p=0.5636).

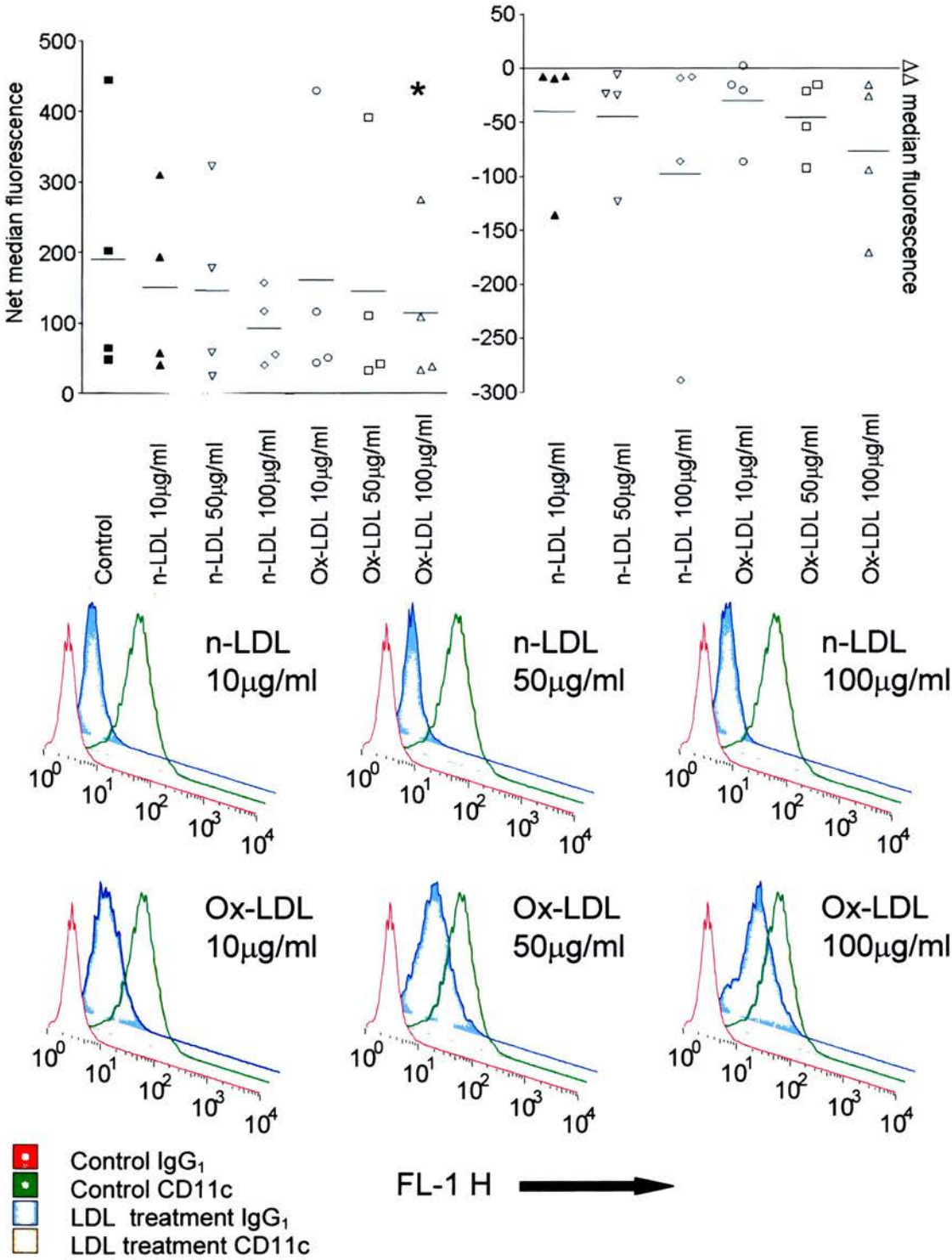


Figure 6-39 Monocyte CD11c, suspension culture, 72 hours, LDL supplements

A reduction in total monocyte surface expression of CD11c was noted after 72 hours in 100µg/ml ox-LDL (one-way ANOVA, n=4, p=0.0281), but did not change relative to control (n=4, p=0.1485). Histograms are offset to illustrate overlaying data plots.

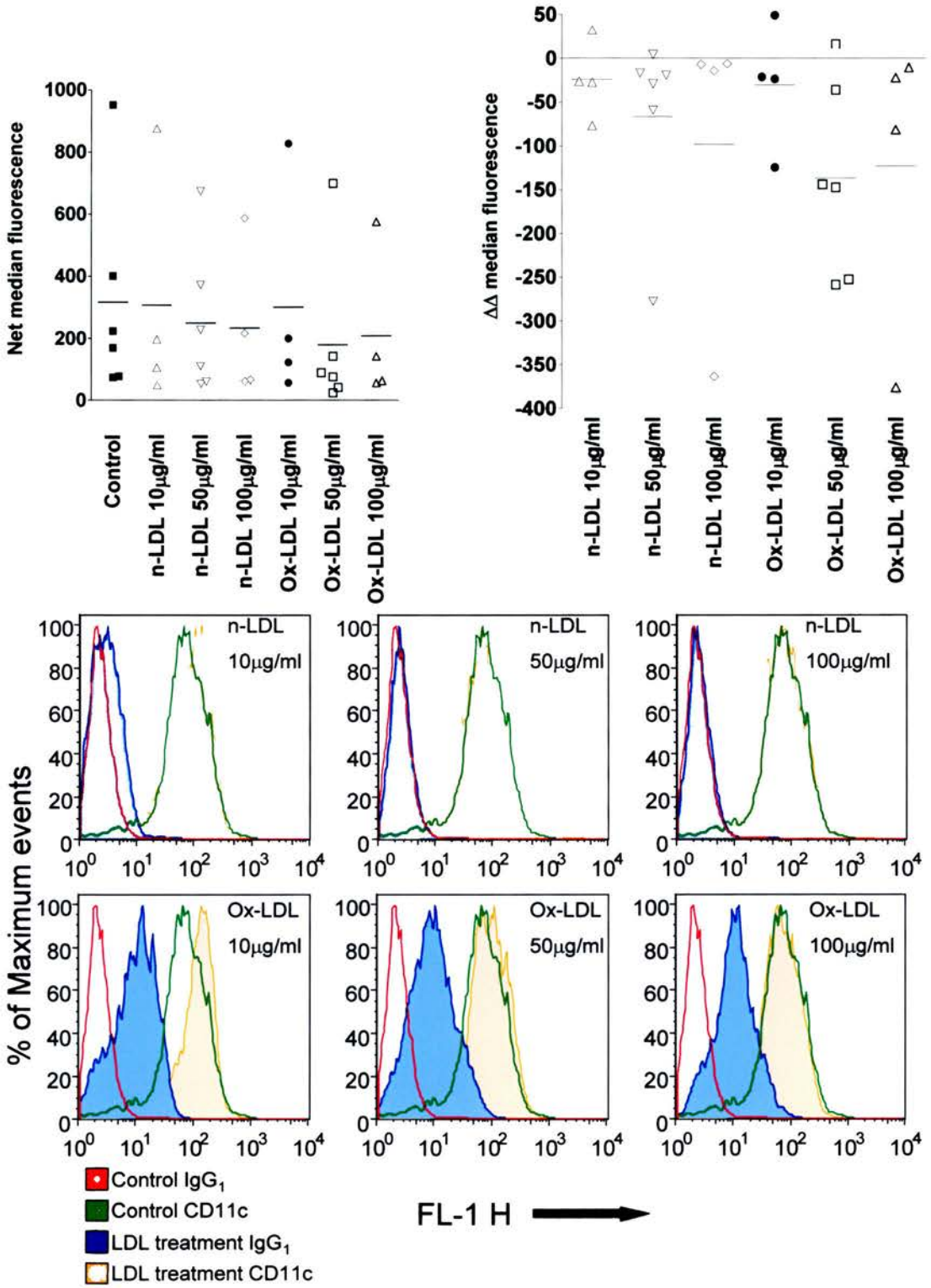


Figure 6-40 Monocyte CD11c expression, suspension culture, 5 days, LDL supplements

No changes in net median CD11c expression (n=4 to 6, p=0.1676) or net fluorescence relative to control (n=4 to 6, p=0.2926) were noted following prolonged suspension culture in the presence of LDL supplements.

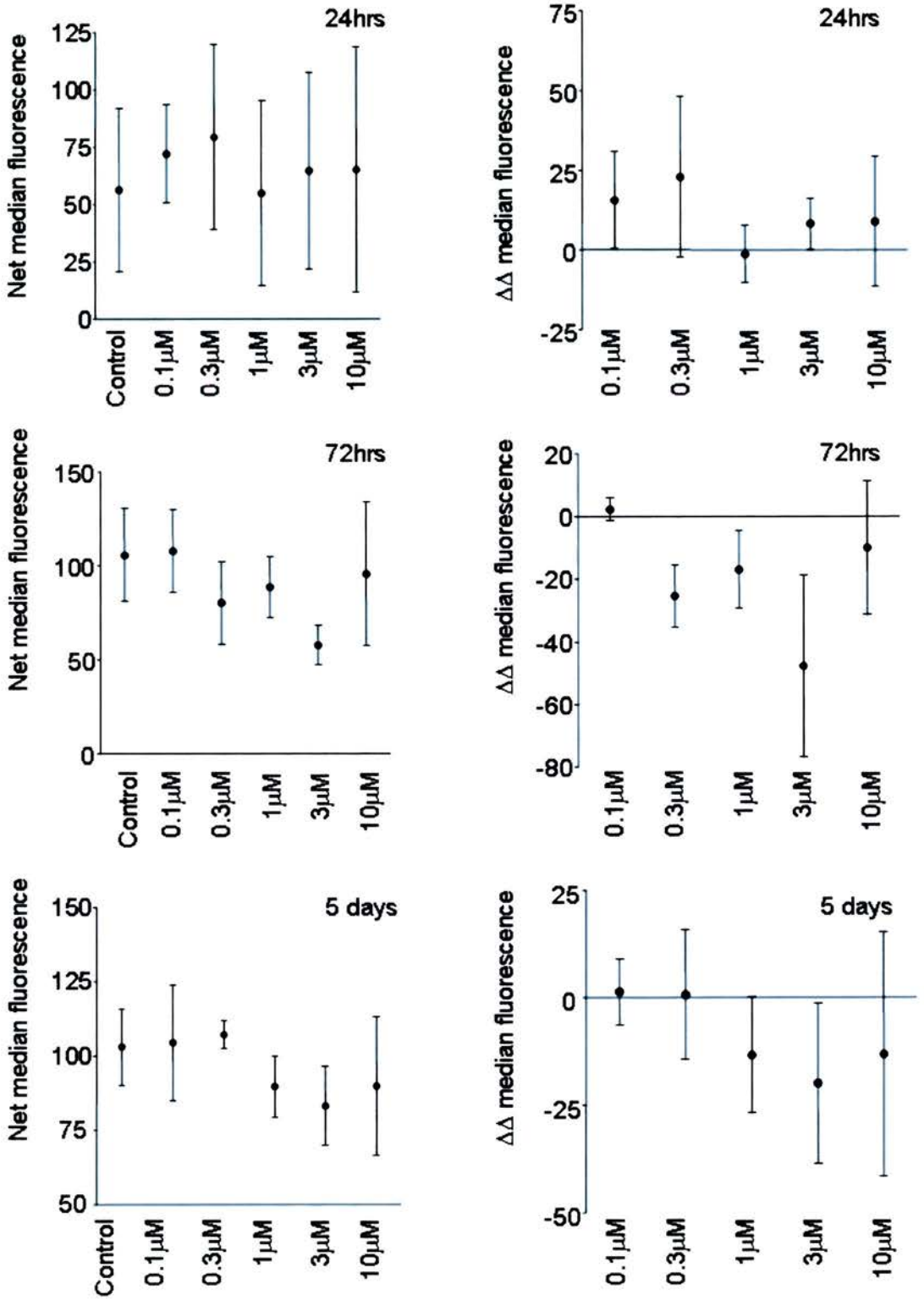


Figure 6-41 Monocyte CD11c expression, suspension culture, 15dPGJ$_2$ supplementation.

No significant alterations in net median CD11c expression (left hand panels) or CD11c expression relative to control (right hand panels) were noted during prolonged culture with 15dPGJ$_2$ at increasing concentrations over a 3-log concentration range (n=3, all non-significant. See data tables for detailed analysis).

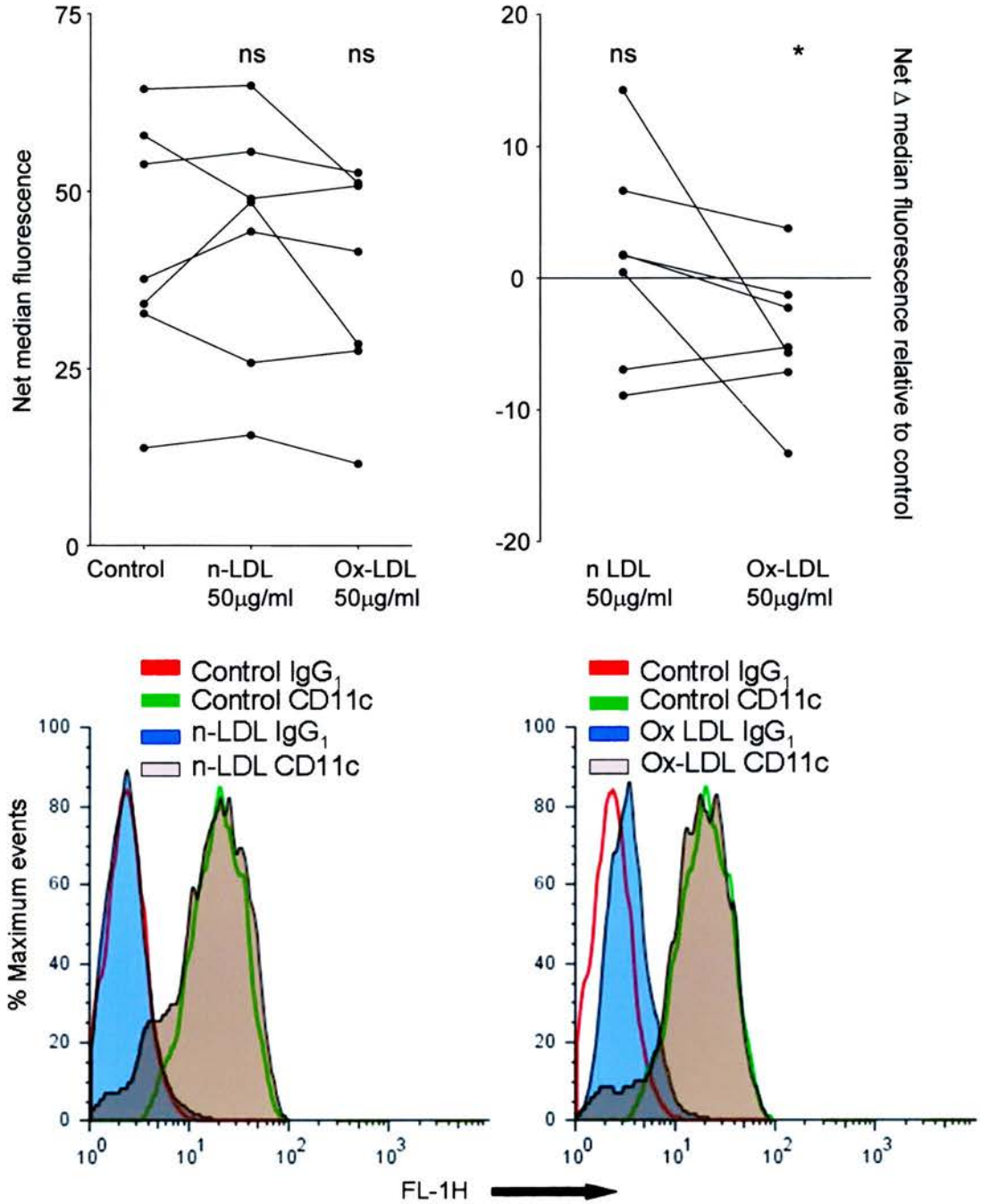


Figure 6-42 Monocyte CD11c expression, 48hrs, adherent culture, LDL supplements.

Net median fluorescence was unchanged by LDL treatments (n=7, p=0.1916 upper left panel), but fluorescence relative to control was reduced following ox-LDL treatments (n=7, p=0.0391, upper right panel). However, representative histogram overlays for CD11c following exposure to n-LDL (lower left panel) and ox-LDL (lower right panel) at 50μg/ml do not reflect this.

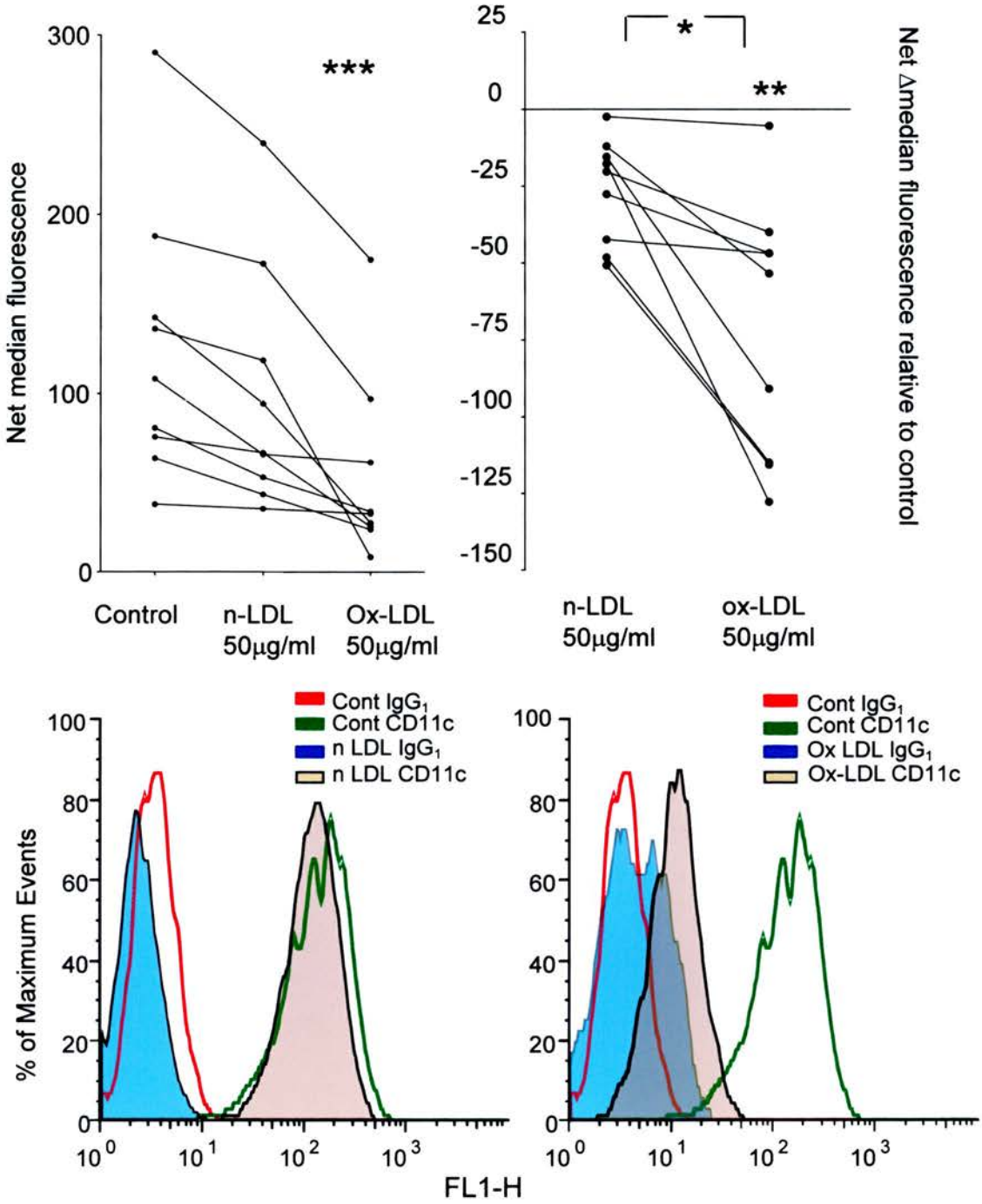


Figure 6-43 Monocytes CD11c surface expression, day 8, adherent culture, LDL supplements

Ox-LDL mediated statistically significant reductions in net median CD11c fluorescence (one-way ANOVA, n=9, $p<0.0001$, upper left panel) and CD11c fluorescence relative to control (n=9, $p=0.002$, upper right panel) in mature monocytes. A significant difference in relative fluorescence between n-LDL and ox-LDL was noted (n=9, $p=0.0244$, upper right panel). Representative histograms illustrate differential effects of ox-LDL and n-LDL.

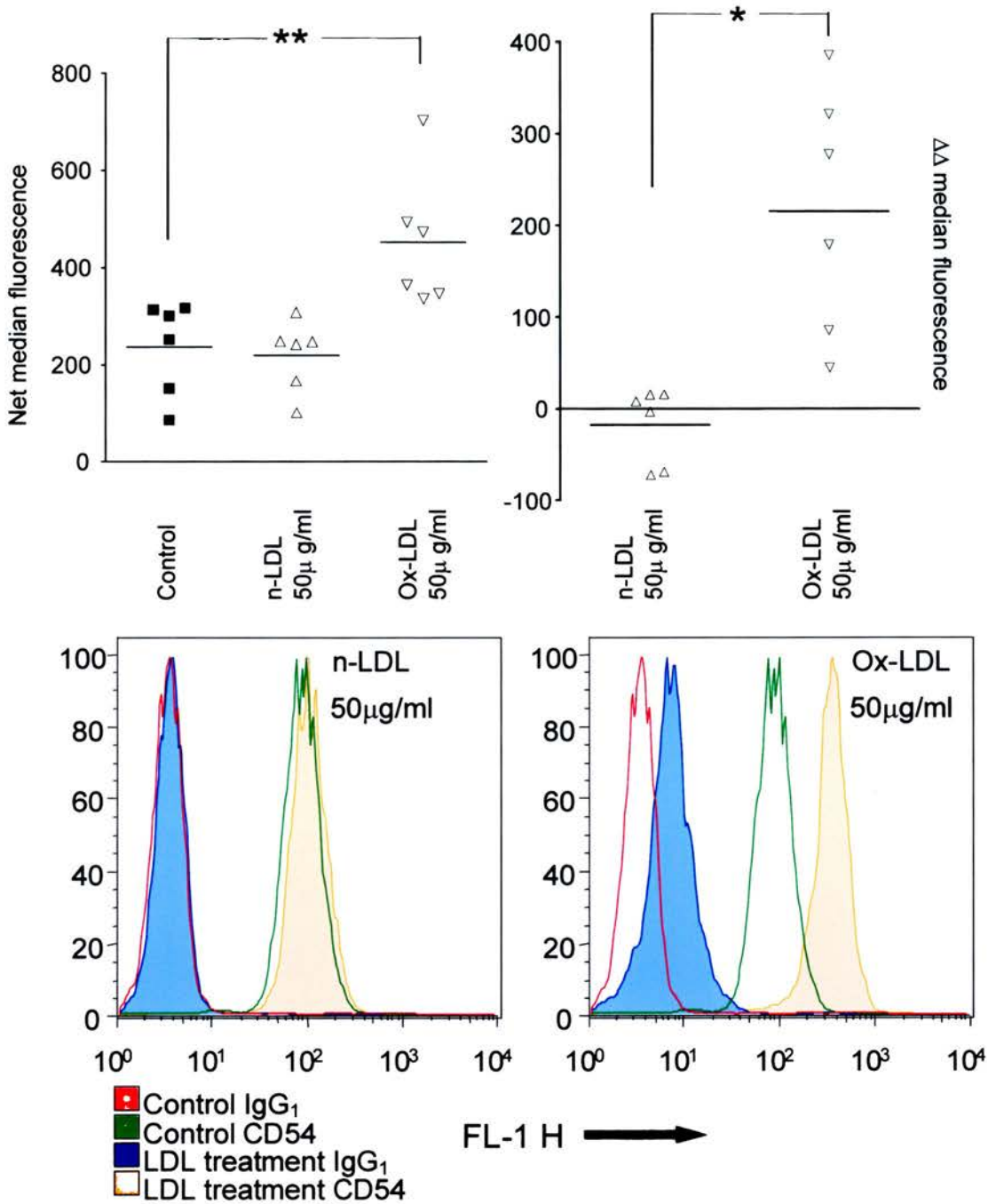


Figure 6-44 Monocyte CD54 surface expression, 24hrs, suspension culture, LDL supplements

Monocyte CD54 expression is significantly elevated by Ox-LDL at 24hrs (total net fluorescence). No corresponding changes following n-LDL exposure were seen. This increase was reflected in total fluorescence levels (one-way ANOVA, p=0.0081, n=6), as well as net changes in fluorescence relative to control (Wilcoxon signed rank test, p=0.0156, n=6).

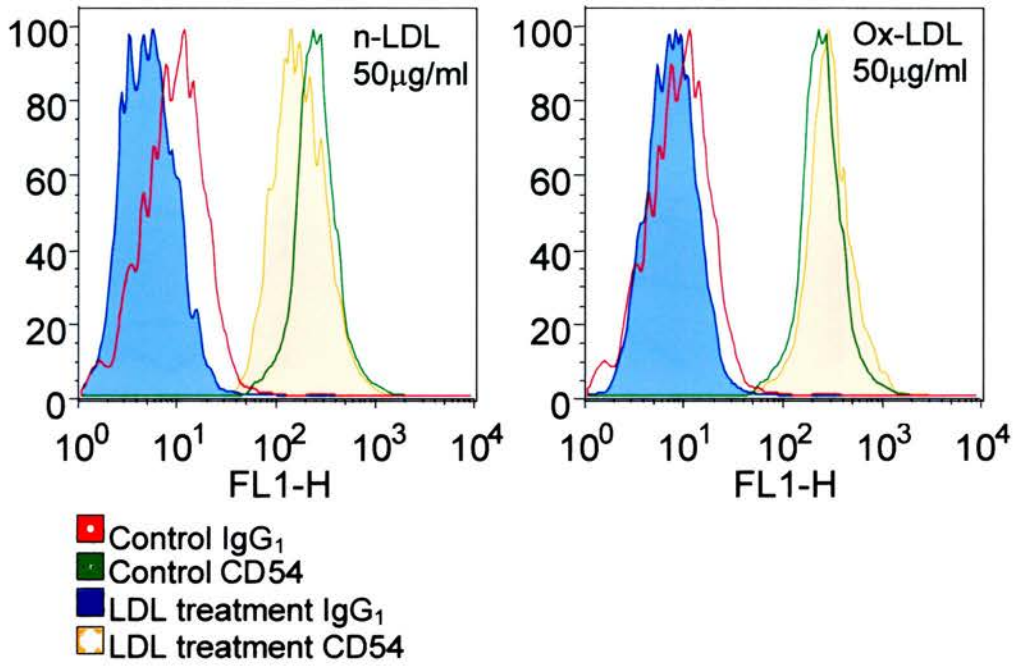


Figure 6-45 Monocyte CD54 expression, suspension culture, 5 days, LDL supplements

Insufficient samples were available to statistically assess CD54 expression on suspension culture cells at late time points. It appeared on limited phenotyping that CD54 expression did not differ with LDL treatments from control.

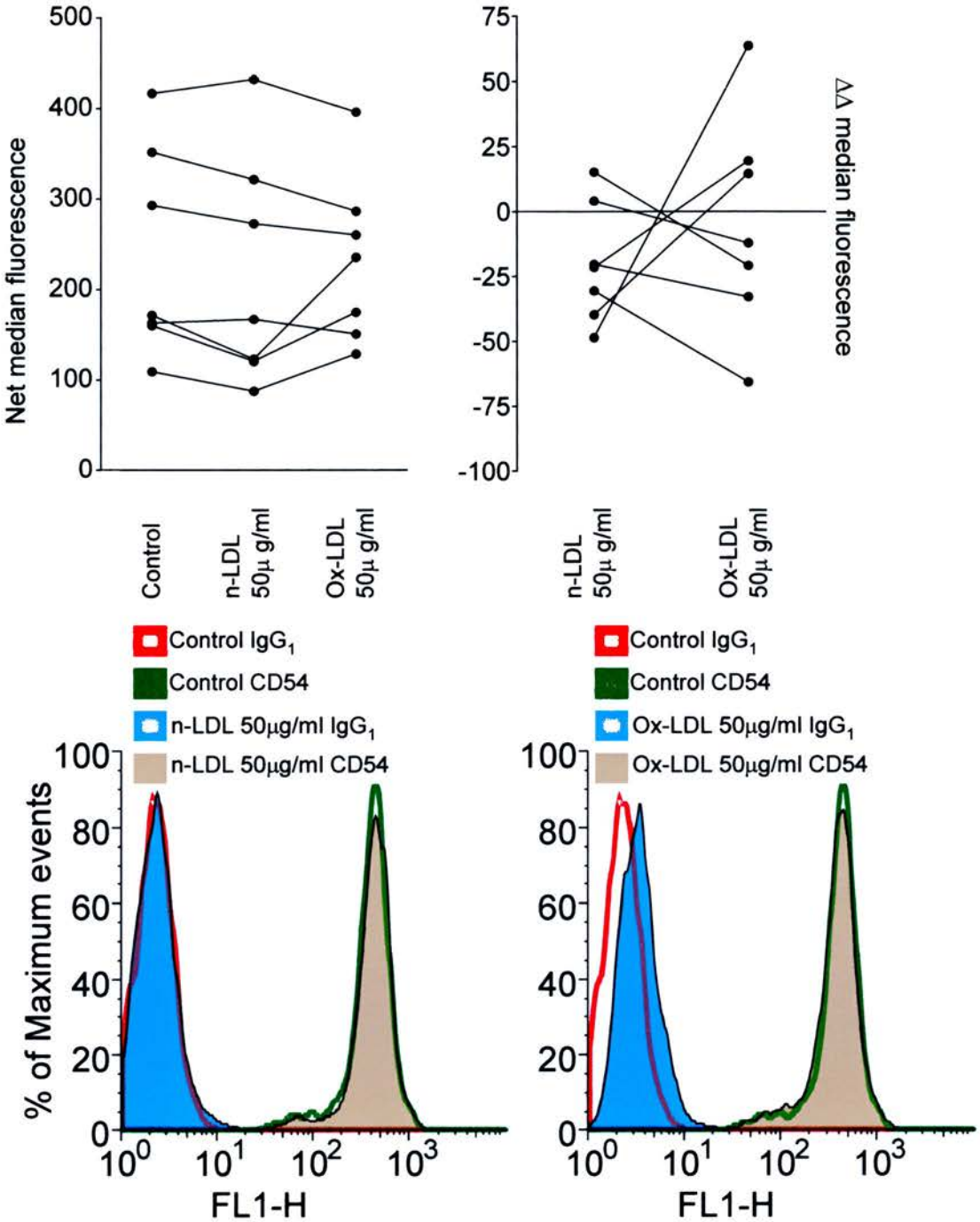


Figure 6-46 Monocyte CD54 expression, 48hrs, adherent culture, LDL supplements

No significant changes in net median CD54 expression (one-way ANOVA, n=7, p=0.7682, upper right panel) or net median fluorescence relative to control (Wilcoxon signed rank test, n=7, p=0.3438, upper right panel) were seen during early adherent culture following LDL exposure. No differences in relative expression were noted between n-LDL and ox-LDL treatments (n=7, p=0.4557).

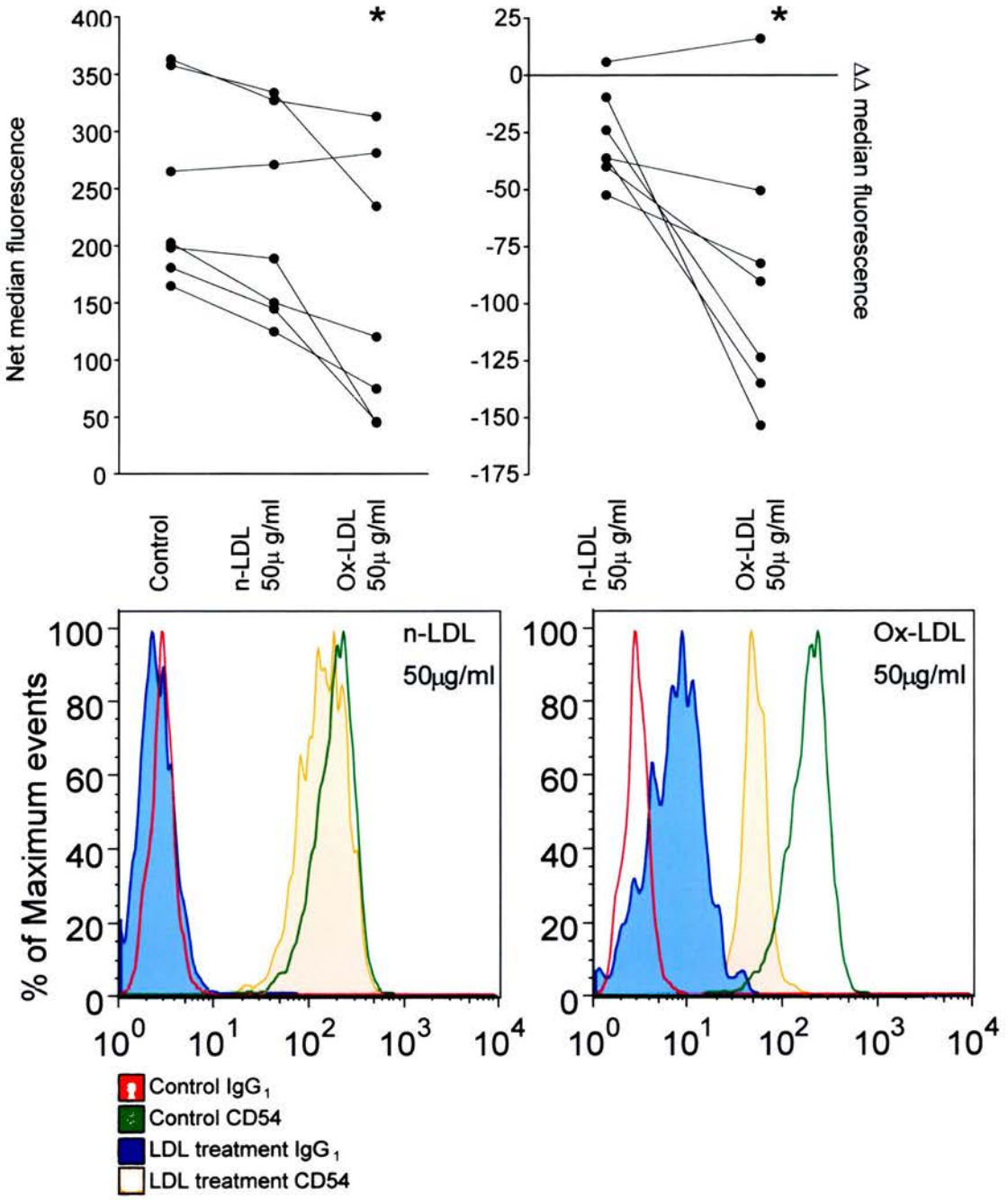


Figure 6-47 Monocyte CD54 expression, adherent culture, 8 days, LDL supplements

Monocyte surface CD54 expression was significantly reduced by exposure to Ox-LDL at 50mg/ml in adherent culture, but not by n-LDL. Reductions for both net median fluorescence (one-way ANOVA, n=7, p=0.0272) and net fluorescence relative to control (Wilcoxon ranked sign test, n=7, p=0.0379) followed ox-LDL exposure.

Chapter 7 CONCLUDING REMARKS

7.1 Overview

Atherosclerosis is a process of chronic inflammation and repair in which monocytes play a central co-ordinating role, directing injurious and reparative response in the vessel wall. The work presented in this thesis assesses the role that micro-environmental influences, relevant to both inflammation and atherosclerosis, play in monocyte survival, gene transcription, and phenotypic change. This concluding chapter will address how these data relate to our current and future understanding of inflammation and atherosclerosis, in the context of both biological mechanisms and potential therapies.

7.2 Monocyte apoptosis overview

The survival responses of monocytes to ox-LDL and cyPGs noted in Chapter 3 are relevant to leukocyte turnover in the inflamed vessel wall. The *in vitro* induction of monocyte apoptosis serves not as a model for *in vivo* vascular events, but as a means of assessing whether individual pro-inflammatory and pro-atherogenic stimuli may alter leukocyte lifespan.

7.2.1 Lipoprotein oxidation status: relation to cell death & potential therapies

Clinical trials of anti-oxidant therapies including Vitamin E, beta-carotene and alpha-tocopherol, aimed at reducing LDL oxidation and free-radical mediated cell death, have had at best neutral outcomes. Data showing that anti-oxidant therapies reduced the efficacy of cholesterol lowering therapies caused some critics to call into question the role of ox-LDL in the pathogenesis of atherosclerosis (Brown et al. 2001). The role of oxidised LDL as a pathogenic factor in atherosclerosis derives from multiple observations in human disease and animal models of atheroma (Boullier et al. 2001; Navab et al. 1995; Sasahara et al. 1994). Macrophage lipid accumulation is facilitated by LDL modification (Brown & Goldstein 1983), demonstrating the role of macrophage scavenger receptors (Endemann et al. 1993). Further early data highlighted the cytotoxicity of ox-LDL (Hessler et al.). Changes in monocyte mitochondrial potential during free cholesterol loading of macrophages (Yao & Tabas 2001), and direct CD36 ligation by ox-LDL particles (Wintergerst et al. 2000) have both been postulated to act as pro-apoptotic stimuli (see Chapter 3). The rate constant of Vitamin E with superoxides is 6 log orders lower than that for interactions between vascular nitric oxide and superoxide, suggesting that Vitamin E is an inappropriate means of reducing oxidative stress (Landmesser & Harrison 2001). However, this does not detract from the role

that oxidative stress may play in vascular damage; the complex effects that oxygen species have upon leukocytes and vascular wall components suggest that clinical trials of anti-oxidants have been based on an over-simplistic understanding of the biological processes involved (Steinberg & Witztum 2002). Data from murine models of atherosclerosis with 12/15 lipoxygenase gene deletions prove these enzymes are critical in inducing lipoprotein oxidation within the vessel wall (Cyrus et al. 2001). 12/15 eicosanoid products of lipoxygenases are further elevated in murine models of diabetic atherosclerosis, contributing to enhanced monocyte/endothelial adhesion (Hatley et al. 2003). Targeting lipoxygenases within the vessel wall may thus be a more relevant goal for therapies, as a reduction of pro-inflammatory lipoprotein oxidation products might reduce local vascular injury. The recent discovery that terpenoids and chromanes isolated from marine sponge organisms act as potent recyclable inhibitors of human 15 lipoxygenase may provide novel routes for future atherosclerosis treatments (Cichewicz et al. 2004).

7.2.2 PPARγ and monocyte apoptosis

PPARγ is an important nuclear receptor in atherosclerosis, with documented effects upon atherogenesis and inflammation as well as controlling cellular insulin sensitivity. However, no direct link between PPARγ and monocyte apoptosis appears likely (Chapter 3) contradicting data from other laboratories that have reported PPARγ-mediated programmed cell death in monocytes or monocytic cells (Chinetti et al. 1998; Kawahito et al. 2000).

The use of high-affinity thiazolidinedione PPARγ ligands in current clinical practice is widespread within diabetes and cardiovascular medicine (Sidhu & Kaski 2001). Despite concerns regarding PPARγ ligation promoting unregulated lipid uptake in leukocytes (Nagy et al. 1998; Tontonoz et al. 1998), synthetic PPARγ agonists in animal models of atherosclerosis have shown a beneficial modulation of atherosclerotic plaque load (Duval et al. 2002; Hsueh & Law 2001; Li et al. 2000). Furthermore, increased levels of atherosclerotic complications or innate immune compromise have not been reported in clinical studies of patients receiving PPARγ agonists, suggesting that neither lipid-uptake nor dramatic leukocyte cell death follow from the systemic administration of these agents (Wang & Tafuri 2003).

The indications that NF-κB was involved in cyPG mediated apoptosis fits with known pro-survival effects of cellular activation, and have already been discussed in Chapter 3. More intriguingly, the mechanisms of ox-LDL induction of apoptosis are less clear. In separate experiments, monocyte exposure to LDL appeared to induce I-κB degradation for both native and oxidised LDL (data not shown). It is highly likely that distinct mechanisms of cell

death induction operate for ox-LDL and cyPGs, but neither agent induces apoptosis *via* PPARγ.

Increasing evidence suggests that ligands for PPARγ limit inflammatory responses *via* PPARγ independent mechanisms (Chawla et al. 2001a). PPARγ modulation of inflammatory responses is mediated by inhibiting NF-κB target genes, but not NF-κB mediated cell activation *per se*. The complex mechanics of this involve covalent attachment of the small ubiquitin-related modifier (SUMO) to the PPARγ ligand-binding domain, which targets PPARγ to nuclear receptor co-repressor (NCoR)-histone deacetylase-3 (HDAC3) complexes on inflammatory gene promoters. SUMOylation prevents recruitment of the ubiquitin/19S proteosome that mediates the removal of co-repressor complexes required for gene activation (Pascual et al. 2005), inhibiting PPARγ transcriptional activity. Contrary to previous models of PPARγ driving foam cell generation, evidence suggests that PPARγ acts to moderate lipid uptake, controlling foam cell formation (Li et al. 2004). More recent data using conditional PPARγ macrophage gene deletion in murine models have demonstrated increased atherosclerosis (Babaev et al. 2005). This suggests that PPARγ ligation by thiazolidinediones is not detrimental in atherosclerosis therapies. PPARα may be more important in driving macrophage lipid loading and this may explain the therapeutic benefit of fibrates in clinical practice (Vosper et al. 2002).

7.2.3 Monocyte apoptosis: implications for atherogenesis and plaque progression

Apoptosis is present at all stages of atherosclerosis (DeVries-Seimon et al. 2005), with distinct outcomes ensuing from cell death at different stages of the disease process (see Chapter 3 section 3.4.4.). Even within individual plaques, cell surface markers demonstrate heterogeneity (van der Wal et al. 1992), with macrophages close to the endothelium displaying altered features of maturation and differentiation, affecting susceptibility to apoptosis as well as phagocytic capability. The role of apoptosis in early vs. advanced plaques has already been discussed (Chapter 3), with special reference to plaque progression and stability (Kolodgie et al. 2000) and thrombosis (Hutter et al. 2004). Alterations in cell death contribute to the gross histological changes reviewed in Chapter 1, including both necrotic core formation in advanced lipid rich atheromata (Geng & Libby 1995), and the hypocellularity noted in advanced fibrotic lesions (Kockx 1998).

Plaque volume is not static, and clinical evidence shows that it may be reduced following aggressive lipid-lowering treatment with HMG CoA reductase inhibitors (Nissen et al. 2005). *In vitro* data has suggested that blocking cholesterol transport using an acyl-

coenzyme-a:cholesterol acyltransferase (ACAT) inhibitor might induce monocyte-derived foam cell apoptosis and contribute to lesion regression (Rodriguez, Bachorik, & Wee 1999). More recent data using ACAT gene deletions, suggest that this strategy so disrupts cholesterol efflux, that atherogenesis is promoted, despite increased intra-lesional apoptosis (Dove et al. 2005). Although established lipid lowering therapies offer significant benefits, it is worth considering further options that might beneficially target cell survival in atherosclerosis.

The data presented in this thesis cannot be directly extrapolated in order to speculate upon monocyte behaviour in human atherosclerotic plaque. However, the susceptibility of monocytes to both cyclopentenones and ox-LDL suggest that local effects that may pertain to atherosclerotic lesions are able to affect monocyte survival, with potential consequences for lesion volume and local inflammation.

7.2.4 Potential therapies to manipulate apoptosis in atherosclerosis

The demonstration that ox-LDL alters apoptosis raises the possibility that monocyte apoptosis may be manipulated pharmacologically, with relevance to vascular injury mediated by LDL, mechanical trauma, and systemic inflammation. It is arguable that pro-apoptotic agents might aid lesion resolution at the fatty streak stage. Parallels might be drawn with the development of anti-neoplastic therapies which have used inhibitors of Bcl-2, Bcl-xl and Bcl-w proteins to facilitate tumour regression (Oltersdorf et al. 2005). However, the diffuse nature and heterogeneity of atherosclerosis limits the general applicability of pro-apoptotic treatments, which could potentially induce multiple adverse events. Systemic pro-apoptotic therapies may cause unacceptable levels of cell death that may not be obviated by targeting specific cell types. For example, promotion of leukocyte apoptosis would have the potential to breach innate immune defences. Promoting VSMC apoptosis might reduce fibro-reparative responses and arterial wall strength, mimicking inflammatory events following pathological plaque erosion or fissuring (Boyle et al. 2001), and might be detrimental in advanced atherosclerotic plaque. Local intra-vascular delivery of pro-apoptotic drugs introduces the prospect of inducing targeted cell death in VSMCs and ECs, initiating inflammatory responses and enhancing vascular damage. Cell cycle inhibitors, originally assessed as immunosuppressant therapies in vascular disease (Gregory et al. 1995), are successfully used as a focal intervention in coronary disease by limiting cellular hyperplasia and neo-proliferation, rather than invoking indiscriminate cell death (Serruys, Kutryk, & Ong 2006). An example of more novel therapeutic approaches to inducing focal apoptosis is the use of targeted photodynamic therapies that introduce photo-excitable molecules such as

fullerenes into cells. Illumination induces reactive oxygen species production, and photo-sensitisers taken up *via* scavenger receptors may allow the selective induction of cell death in macrophages (Demidova & Hamblin 2004), with potential for catheter based interventions when treating focal atherosclerosis.

Prevention of monocyte/macrophage apoptosis might be beneficial in stabilising advanced atherosclerotic lesions, but would not address the issues of cell accumulation increasing plaque bulk, nor reduce the possibility that macrophages permitted to survive would still be capable of pro-inflammatory responses, proteolytic degradation and the induction of VSMC apoptosis.

7.2.5 Potential therapies to manipulate phagocytosis in atherosclerosis

Altered phagocytic clearance of apoptotic cells has been noted in murine models of atherosclerosis bearing apoE gene deletions (Grainger, Reckless, & McKilligin 2004). Furthermore, murine models bearing gene deletions for both FasL and apoE have high levels of circulating un-cleared apoptotic bodies, suggesting that phagocytic clearance capacity has been exceeded in this model of accelerated atherosclerosis (Aprahamian et al. 2004). Phagocytosis is further reduced in this model by the infusion of ox-LDL or lysophosphatidic acid, and is associated with a low level of uptake of apoptotic cell debris by phagocytes. Thus, in the setting of already elevated levels of apoptosis, optimising phagocytic clearance may be a safer strategy for developing new anti-atherosclerotic therapies.

Paradoxically anti-oxidant therapies may limit effective phagocytosis and therefore targeted inhibition of oxidation may alter clearance of apoptotic cells. Etoposide, a lipid anti-oxidant and anti-tumour agent that is capable of inducing apoptosis, has been shown to limit phosphatidylserine (PS) oxidation, thus reducing PS exposure and apoptotic cell recognition (Tyurina et al. 2004). Promotion of phagocytosis would enable rapid resolution of chronic inflammatory vascular injury, ensuring that response to retained lipid were not injurious. The use of gluocorticoids in promoting clearance have been closely investigated (Liu et al. 1999), and are of clinical benefit in many inflammatory situations (Haslett 1992). However, gluocorticoids induce vascular pathology, and are pro-diabetic and atherogenic (del Rincon et al. 2004), precluding their use in atherosclerosis treatments. Glucocorticoid products such as Annexin I may be of use as an alternative, to promote phagocytosis. Lipoxins and epilipoxins form during inflammatory responses and are present in atherosclerotic lesions, and may alter granulocyte activation (Qiu et al. 2001). Macrophage phagocytosis has been reported to be enhanced using lipoxins (Godson et al. 2000; Reville et al. 2006) and epilipoxins (Mitchell et al. 2002).

The phagocytic defect in apoE null mice was noted to be restored by the infusion of apoproteins (Grainger, Reckless, & McKilligin 2004), suggesting a potential mode of novel pro-phagocytic therapy using a small peptide molecule or amino-acid peptide mimetic agents bearing similar structures to apoproteins. However, it is unclear what effect this may have upon atherosclerotic plaque bulk and stability.

7.3 Use of gene arrays in examining monocyte transcriptional profiling

Transcriptional profiling is increasingly used to measure biological responses and can offer initially very rapid screening assays to examine cellular changes after defined stimuli, as discussed in Chapter 5. Their use is limited by experimental design, in particular the need to minimise the number of experimental variables. Transcriptional profiling does have a place in the clinical assessment of complex chronic inflammatory processes such as atherosclerosis. RNA sampling from clinical subjects for gene array analysis has been successfully undertaken using explanted human coronary tissue (Satterthwaite et al. 2005). More novel profiling techniques such as the use of single nucleotide polymorphism profiling arrays (Syvanen 2005) may help identify risk factors associated with clinical disease enabling further information to complement *in vitro* work to elucidate candidate genes involved in atherosclerotic plaque progression.

7.4 Monocyte phenotypic changes

Monocyte/macrophages within atherosclerotic plaque differ phenotypically from circulating monocytes (van der Wal et al. 1992). Alteration of monocyte phenotype affects function, (see Chapter 6), with impact upon the uptake of lipoproteins and apoptotic cells *via* scavenger receptors, as well as innate and adaptive immune capacity and macrophage motility.

7.4.1 Immune mechanisms in vascular disease

The involvement of innate immune mechanisms in atherosclerosis has received increasing attention (Binder et al. 2002). Pattern-recognition receptors such as scavenger receptors and toll-like receptors (TLR) are able to recognise pathogen-associated molecular patterns that may be shared by modified lipoproteins pathogens as well as apoptotic cells. Furthermore, the acute phase reactant pentraxin-structured C-reactive protein has been shown to bind ox-LDL and oxidised phosphatidylcholine, with specific binding of CRP to apoptotic Jurkat T cells (Chang et al. 2002). The lack of change in both scavenger receptor expression and in surface molecules related to immune function was thus surprising (Chapter 6). Multiple reports have suggested that the chronic inflammatory stimuli that propagate vascular injury

may in part be mediated by specific immune pathways (reviewed by Hansson et al. 2002). Class II MHC is critical to antigen presentation, and is highly expressed in human atherosclerosis, with further increases noted in post-mortem studies of diabetes-associated atherosclerosis (Burke et al. 2004). As discussed in Chapter 6, it is likely that the model of monocyte LDL exposure is too simplistic to allow discernment of changes in adaptive immunity without embarking upon co-culture work, a criticism that applies equally to the phenotypic analysis of B7 co-stimulatory molecules.

Monocytes from patients suffering acute coronary syndromes show elevated transcription of TLRs as well as B7 co-stimulatory molecules (Methe et al. 2005). Early innate immune responses to microbial pathogens are mediated by TLRs, which initiate NF-κB activation *via* the adapter protein MyD88 (Schnare et al. 2001). Macrophage TLR-4 expression is elevated in aortic atheromatous plaques in apoE-/- mice, and from *ex vivo* human atherosclerotic samples, localising to plaque shoulder regions and the periphery of the necrotic core (Xu et al. 2001), suggesting that monocyte TLRs merit attention in further studies of monocyte behaviour in plaque. Ischaemia reperfusion injury models highlight a further role of B7 molecules with relevance to atherosclerosis. Blockade of B7 molecules in ischaemia reperfusion injury during organ transplantation has been associated with lower levels of lymphocytic infiltration and enhanced tissue viability (Takada et al. 1997). B7 blockade using a fusion protein made of the competitive CD28 homologue CTLA4 and the IgG_1 heavy chain limits chronic cardiac allograft rejection and limits the progression of transplant accelerated arteriosclerosis (Russell et al. 1996).

The finding that antibodies specifically recognise ox-LDL (Horkko et al. 1999) and cross-react with apoptotic cell membrane-derived phosphorylcholines (Shaw et al. 2000) remains a focus of interest. The fact that antibody cross-reactivity also extends to bacterially derived phosphorylcholines (Binder et al. 2003) suggests that responses to lipid oxidation may represent conserved mechanisms of pathogen recognition that have been subverted in atherosclerosis (see Chapter 1 and Chapter 3). Although experiments presented here and by other investigators have used copper-oxidised LDL is a heterogeneous source of ox-LDL epitopes, the immuno-dominant epitope in ox-LDL is oxidised phosphorylcholine (Friedman et al. 2002), suggesting that this particular lipoprotein stimulus is a valid means of assessing monocyte responses.

A detailed discussion of the role that immune therapies might play in atherosclerosis is beyond the scope of this thesis. Nonetheless the possibility of using vaccination strategies to limit atherosclerosis is intriguing. Data from murine models suggests that atheroprotective effects may follow the generation of predominantly B-cell driven anti-ox-LDL IgM

antibodies (Palinski, Miller, & Witztum 1995), with derivatives of ox-LDL, particularly malondialdehyde (MDA), acting as the epitope (Freigang, Witztum, & Palinski 1998). Parallel experiments have suggested that immunisation against MDA-apoprotein B-100 adducts may also confer atheroprotection, *via* T cell-dependent IgG production (Schiopu et al. 2004). Further insights into immune mechanisms governing atherosclerosis may thus offer novel non-pharmacological therapies.

7.4.2 Changes in macrophage mobility

Responses to ox-LDL may alter macrophage mobility (Terasawa et al. 2000), with effects upon movement across the vessel wall (Weerasinghe et al. 1998). Ox-LDL has been shown to affect leukocyte motility at a molecular level by reducing F-Actin polymerisation, a key step in cytoskeletal rearrangement (Miller et al. 2003), potentially affecting trans-endothelial migration (TEM).

Alteration in leukocyte TEM is relevant to monocyte recruitment and emigration throughout atherosclerotic plaque development, and is tightly regulated by phosphorylation cascades including ephrin tyrosine kinases (Aasheim, Delabie, & Finne 2005), and Wiskott-Aldrich syndrome protein (Cory et al. 2002). In early lesions, intimal fatty streak formation is not matched by the development of lymphatic channels or vasa vasorum (Chapter 1), limiting the exit routes available for infiltrating leukocytes. Advanced plaques have developed these vascular channels in atherosclerotic vessel walls, although they do not directly communicate with the resident intimal leukocyte population. Adventitial angiogenesis does not initiate intimal thickening but may help accelerate intimal changes (Khurana et al. 2004). Inhibition of angiogenesis can alter neo-intimal hyperplasia and limit intimal plaque formation in murine atherosclerosis (Moulton et al. 1999) with more recent data suggesting that leukocyte influx to arterial walls may be dependent upon new adventitial vessels (Moulton et al. 2003). Electron microscopy data showing monocytes re-entering the arterial lumen from the vessel wall has been revisited in the context of reverse transmigration patterns (Randolph et al. 1998). The phenotype that lipid laden macrophages display (comprising of reduced CD11b and CD11c but stable CD14, see Chapter 6) may contribute to limited cell motility. An inverse phenotype of high CD11b and CD11c expression is seen in the dendritic cell (DC)-like cells that reverse transmigrate across endothelial monolayers (Muller & Randolph 1999), and in similar cells that have been shown to exit atheroma *in vivo* (Llodra et al. 2004). The reductions in adhesion molecule expression reported in Chapter 6 are unlikely to represent a homeostatic mechanisms limitation of lipid-laden macrophage binding to the vascular wall. Indeed downregulation of CD49d, CD54 and β2 integrins appears to be linked

to maturation and adhesion. Although the gene array data (Chapter 5) suggested early increases in a number of integrins including CD11b and β3 chains, flow cytometric analysis showed that surface expression of these molecules was either unchanged, or downregulated. High levels of integrin subunit stability at the cell membrane may explain this, or the fact that monocyte transcription alters through prolonged exposure to lipids, a possibility that could be answered by more extensive profiling over multiple time points.

If the phenotype induced by ox-LDL is also seen in non-reverse transmigrating cells, and more importantly in cells remaining in plaque, this may be an indication of why monocytes cannot leave atherosclerotic lesions. Furthermore, a rescue of the original phenotype might be worth pursuing, using lipid withdrawal, or examining anti-oxidant strategies.

Monocyte-derived DCs in apoE null mice display reduced mobility induced specifically by PAF and ox-LDL. DC emigration can be restored in this model by the intravenous administration of exogenous HDL, due to the presence of PAF acetylhydrolase (Angeli et al. 2004). Current systemic lipid lowering therapies including HMG CoA reductase inhibitors ("statins") are widely used to reduce cardiovascular risk. Recent data suggest that statins may act to reduce monocyte CD54 and CD40 expression, decreasing adhesion and inflammatory signalling (Takahashi et al. 2005). Furthermore, statins and derivative agents may directly alter adhesion molecule expression and behaviour. CD11a/CD18 integrin binding to CD54 is limited by allosteric inhibition of a novel binding site in the insertional domain (Weitz-Schmidt et al. 2001), and expression of CD54 and CD11a was down-regulated on circulating monocytes in a study of hypercholesterolaemic patients receiving simvastatin (Rezaie-Majd et al. 2003). Altered adhesion molecule expression may thus be clinically relevant, and the development of drugs targeting monocyte adhesion molecules may provide future therapies for atherosclerosis.

Therapies targeting adhesion molecules are now available for other inflammatory diseases. Efaluzimab, a humanised IgG_1 monoclonal antibody that blocks CD11a, has been used to treat psoriasis, by blocking T cell recruitment to skin lesions (Lebwohl et al. 2003). The prospect that such an agent might inhibit lymphocyte or monocyte recruitment to atherosclerotic plaque, reducing local vascular inflammation, is intriguing and merits investigation. Recent studies have suggested that a novel anti-oxidant agent with inhibitory effects upon VCAM-1 transcription may beneficially decrease post-barotrauma restenosis following percutaneous coronary intervention (Kunsch et al. 2004). Anti-sense oligonucleotides are able to inhibit adhesion molecule function (Bennett et al. 1994), and ulcerative colitis has been successfully treated in humans by inhibiting CD54-mediated leukocyte adhesion using the anti-sense agent Alicaforsen (van Deventer et al. 2004). This

latter strategy may be of future use in limiting initial monocyte adhesion and thus monocyte-mediated inflammatory damage during acute vascular injury, with relevance to plaque degradation.

7.5 Conclusions

Evidence that pro-atherogenic and pro-inflammatory stimuli alter monocyte survival *in vitro* is presented in this thesis.

Monocytes undergo apoptosis in response to modified lipoproteins and arachidonic acid metabolites. These findings may partially explain locally elevated levels of apoptosis and inflammation in atherosclerotic plaques.

Oxidised low-density lipoproteins reduce the expression of specific monocyte adhesion molecules, in a pattern that may explain the reduced emigration of monocytes from atherosclerotic plaque.

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